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Transcription factor heterogeneity in epiblast pluripotency

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Declaration

I hereby declare that this thesis was composed by me, and the research presented is my own, except where otherwise stated. This work has not been submitted for any other degree or professional qualification.

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Abstract

Pluripotency is the ability of a cell to differentiate into derivatives of all three somatic lineages and germ cells. *In vivo*, pluripotent cells exist transiently in the epiblast of the developing embryo and in rare tumour cells. *In vitro,* pluripotent cells have been isolated and propagated from teratocarcinomas (EC cells), pre-implantation epiblast (ES cells) and post-implantation epiblast (EpiSCs). Pluripotency is governed by a gene regulatory network centred on the triumvirate of transcription factors Oct4, Sox2 and Nanog. Interestingly, transcription factors that are important to direct pluripotent cell identity are not all equally distributed throughout the pluripotent cell population. While Oct4 levels are relatively homogeneous, other transcription factors, such as Nanog, are more heterogeneously expressed. Additionally, an increasing body of evidence indicates that extrinsic cues also play a critical role in the establishment and maintenance of pluripotency.

Using biochemical and genetic tools in mouse ES cells, the role of FGF signaling and Sox2 levels on heterogeneous Nanog expression was examined. Interference with FGF or ERK activity by genetic ablation or signal inhibition, promoted high, homogenous Nanog expression and enhanced self-renewal. This is consistent with reports showing that similar manipulations reduced the ability of ES cells to commit to differentiation. Moreover, ES cells with reduced Sox2 levels displayed greater heterogeneity for Nanog expression than wild-type ES cells.

Pluripotency is lost in the mouse embryo around E8.5, however, the precise timing and mechanism involved in this process has not yet been defined. Here it is shown that pluripotency is extinguished at the onset of somitogenesis, coincident with reduced expression and chromatin accessibility of Oct4 and Nanog regulatory regions. Prior to somitogenesis, the expression of both Nanog and Oct4 is regionalized. Interestingly, pluripotency tracks the *in vivo* level of Oct4, this correlation does not hold true for Nanog. However, Nanog expression reports on pluripotent cells. Indeed, ectopic Oct4 expression in somitogenesis-stage tissue provokes rapid reopening of *Oct4* and *Nanog* chromatin, Nanog re-expression and resuscitation of moribund pluripotency. Competence to re-activate the pluripotency network upon enforced Oct4 expression is gradually lost with the progression of embryonic development.

ES cells and EpiSCs are two distinct pluripotent populations as they show differences in their ability to undergo clonal propagation, re-colonize embryos, growth factor responsiveness, morphology and gene regulatory networks. It is possible to harness this differential growth factor responsiveness to convert ES cells into EpiSCs. Conversely, EpiSC can be reverted back to ES cell pluripotency through the overexpression of a small number of transcription factors. The inter-conversion of ES cells and EpiSCs has been documented, but detailed analyses of the changes that occur during such transitions had not been performed. The current work shows that Nanog levels are critical for the specification of the pluripotent state of the cells. Furthermore, it is shown that orphan nuclear receptor Esrrb is a potent inducer of ES cell pluripotency in EpiSC. Interestingly, Esrrb was able to restore naïve pluripotency in cells genetically depleted of Nanog.

Abbreviations

MEK	Mitogen-activated protein kinase
GSK3	Glycogen synthase kinase 3
2i	GSK3 (CHIR99021)/MEK (PD0325901) dual inhibition
mRNA	messenger RNA
BMP	bone morphogenetic protein
MEFs	Mouse embryonic fibroblasts
ChIP	chromatin IP
cDNA	complimentary DNA
PBS	phosphate buffer saline
dpc	days post coitum
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
DMSO	dimethyl sulphoxide
EC	embryonal carcinoma
EG	embryonic germ
PGC	primordial germ cell
EpiSC	Epiblast stem cell
PI3K	phosphatidylinositol 3-kinase
ES	embryonic stem
ERK	extracellular receptor kinase
FGF	fibroblast growth factor
FGFR	FGF receptor
qPCR	quantitative real-time PCR

RNA	ribonucleic acid
FCS	foetal calf serum
GFP	green fluorescent protein
SDS	sodium dodecyl sulphate
ICM	inner cell mass
iPS	induced pluripotent stem
IRES	internal ribosomal entry site
STAT	Signal Transducer and Activator of Transcription
JAK	Janus Kinase
Klf	Kruppel-like family
TE	trophectoderm
LIF	Leukemia Inhibitory Factor
GRN	Gene Regulatory Network
PrE	Primitive endoderm
TGFβ	Transforming growth factor-β
FAIRE	Formaldehyde-Assisted Isolation of Regulatory Elements

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Chapter 1

Introduction

1.1 Stem cells

Although a consensus has not been reached on a general definition for the term "stem cell", the prevailing view is that stem cells are cells with the ability to undergo long-term self-renewal and that can generate at least one type of specialized descendant. Self-renewal is the division of a cell to produce two daughter cells, out of which, at least one is identical to the parental cell. On the other hand, differentiation occurs when a cell becomes more specialized and losses proliferative capacity. In the majority of the cases, stem cells do not produce a terminally differentiated cell, but instead produce intermediate/progenitor cell(s) that display a more restricted proliferative and differentiation capacity (Gardner and Beddington, 1988; Guo et al., 2010).

1.2 Pluripotency

The ability of a single cell to generate every cell type in the adult organism has been termed as pluripotency (Smith, 2005, 2006). In order to ensure proper growth and differentiation during embryonic development, the timing and location of pluripotent cells must be tightly regulated. *In vivo*, pluripotent cells exist transiently in the epiblast of the developing embryo, germ cells and in rare tumour

cells. The following pluripotent cells have been isolated: embryonal carcinoma (EC) cells, embryonic stem (ES) cells, embryonic germ (EG) cells and post-implantation epiblast stem cells (EpiSCs). These pluripotent cells have the following origins: EC cells were isolated from teratocarcinomas, ES cells from the inner cell mass (ICM) of blastocysts, EG cells from primordial germ cells (PGCs), and EpiSC were derived from the post-implantation epiblast (Brons et al., 2007; Evans, 1972; Evans and Kaufman, 1981; Martin, 1981; Matsui et al., 1992; Resnick et al., 1992; Tesar et al., 2007).

1.3 Pluripotent populations

1.3.1 EC cells

In the 1970s an observation was made that set down the foundation for pluripotent cell biology. This observation was that certain type of tumours, now known as malignant, contained an undifferentiated component able to seed a new tumour when transplanted into a secondary recipient. These original experiments were performed using tumours that spontaneously arise in the testes of mice (Stevens, 1960, 1964; Stevens and Little, 1954). Malignant tumours, also known as teratocarcinomas, are a mass of cells consisting on a chaotic mixture of differentiated derivatives of the three germ layers and undifferentiated stem cells. The stem cells present in teratocarcinomas were named as embryonal carcinoma due to their resemblance to embryonic tissue. Subsequent experiments performing transplantations of pre-gastrulation embryos into kidney capsules or testes demonstrated, that the origin of teratocarcinomas was not restricted to male germ cells (Damjanov et al., 1971; Solter et al., 1970; Stevens, 1968, 1970). Indeed, later it

was shown that the cellular origin of the teratocarcinoma forming potential of pre-gastrulation embryos was the epiblast (Diwan and Stevens, 1976). The formal confirmation that EC cells are pluripotent stem cells was obtained through the observation that their clonal transplantation could generate secondary teratocarcinomas (Kleinsmith and Pierce, 1964). EC cells could be propagated *in vitro* through explant culture, readily generating stable EC cell lines (Finch and Ephrussi, 1967). Moreover, EC cell lines retain the ability for multilineage differentiation in teratocarcinoma assays. Interestingly, when EC cells were reintroduced into pre-implantation embryos these cells were able to contribute to the hosts' tissues, thereby generating chimeric embryos and/or live animals (Brinster, 1974; Papaioannou et al., 1978; Papaioannou et al., 1975). This indicates that prolonged self-renewal of EC cells is not a malignant transformation, but it is rather a tightly controlled process that can be aborted at any point in response to the appropriate differentiation cues. However, the pressure for selective growth advantage during the propagation of EC cells compromises their genetic constitution. Indeed, the majority of EC cells are aneuploid, such genetic aberrations results in restricted differentiation potential and reduced ability to contribute to chimaeras.

The ground-breaking work performed studying EC cells was pivotal for the subsequent isolation of pluripotent cells from the embryo proper. A critical point was the finding that pluripotent cells heavily benefitted by their co-culture with fibroblasts (Martin and Evans, 1975). The same authors later reported the isolation of pluripotent stem cells lines from mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981). Initially, these stem cell lines were believed to be a contamination of

EC cells. However, it soon became apparent that the embryo-derived stem cells were qualitatively different, given the observation that when put back into pre-implantation embryos these stem cells readily contributed to chimaeras and the germline (Bradley et al., 1984). To denote their tissue of origin, these cells were named embryonic stem cells.

1.3.2 Mouse embryonic stem cells

ES cells are a pluripotent population isolated from mice at embryonic days 3.5-4.5 (E3.5-4.5). The tissue of origin of ES cells is the naïve epiblast, which is a cell layer present in the ICM of mouse blastocysts. The derivation and culture of ES cells has been achieved using combinations of cytokines, growth factors, hormones, serum, serum extracts, conditioned media and feeders. Regardless of the preferred culture conditions, stimulation with the leukemia inhibitory factor (LIF) remains a common practice in the routine culture of mouse ES cells. Extrinsic factors used for the propagation of ES cells have been identified for their specific ability to stimulate or protect the gene regulatory network controlling pluripotency. Considerable evidence indicates that ES cell self-renewal and pluripotency is governed by a transcriptional network centred on Oct4, Sox2 and Nanog (Boyer et al., 2005; Chen et al., 2008; Loh et al., 2006). These master nuclear regulators target genes encoding transcription factors, signal transduction components and chromatin-modifying enzymes that promote self-renewal, while suppressing differentiation (Avilion et al., 2003; Chambers et al., 2003; Chambers et al., 2007; Chambers and Tomlinson, 2009; Masui et al., 2007; Mitsui et al., 2003; Nichols et al., 1998; Niwa et al., 2000).

1.3.3 Post-implantation epiblast stem cells

The mouse embryo at the implantation stage is composed of the epiblast and the extra-embryonic tissues. The later consists of the extra-embryonic ectoderm and endoderm, which will give rise to the placenta and yolk sac respectively. The epiblast is a pluripotent derivative of the ICM. After implantation the epiblast undergoes dramatic expansion and morphogenesis, resulting in its transformation from an unstructured cell mass into a columnar epithelium. The post-implantation epiblast is subject to potent lineage specifying signals from adjacent extra-embryonic tissues. Recently it was shown that pluripotent stem cells could be derived from the epiblast layer of post-implantation mouse embryos (Brons et al., 2007; Tesar et al., 2007). When EpiSC were used in embryoid body and teratoma formation assays these cells were shown to be capable of differentiating into derivatives of all three primary germ layers, thereby proving their pluripotent identity (Brons et al., 2007; Tesar et al., 2007). Additionally, EpiSCs express the core pluripotency transcription factors Oct4, Sox2 and Nanog (Brons et al., 2007; Tesar et al., 2007). Despite expressing Oct4, Sox2, and Nanog, differences in the transcription factor circuitry strongly suggests that ES and EpiSC are two distinct pluripotent states (Bao et al., 2009; Brons et al., 2007; Han et al., 2010; Tesar et al., 2007). For example, T-brachyury, a marker of primitive streak and tail bud, is detected at higher levels in EpiSC when compared to ES cells (Tesar et al., 2007). It is therefore possible that some EpiSC populations reflect an *in vivo* state reminiscent of the primitive streak, in which cells are pluripotent but primed for germ layer differentiation. Notably, EpiSCs display culture requirement and properties similar to human ES cells, but different to mouse ES cells (Tesar et al., 2007). Self-renewal of

EpiSCs and hES cells is promoted by the stimulation of TGFβ/Activin/Nodal signaling pathway (Amit et al., 2004; Brons et al., 2007; James et al., 2005; Tesar et al., 2007; Vallier et al., 2009). It has been proposed that pluripotency in hES cells and mouse EpiSC is sustained by activation of Activin/Nodal signaling. Furthermore, Nanog activation has been identified as the mechanisms through which Activin/Nodal signaling promotes pluripotent identity in hES and EpiSC (Greber et al., 2008; Greber et al., 2010; Vallier et al., 2009; Xu et al., 2008). Nanog has been reported to inhibit neural differentiation in hES cells (Vallier et al., 2009). Interestingly, ES cells have no absolute requirement for Nanog in the maintenance of pluripotency (Chambers et al., 2007). Moreover, EpiSCs differ from ES cells in the fact that they have flattened colony morphology, inefficient clonal propagation and a limited capacity for colonising pre-implantation embryos (Brons et al., 2007; Han et al., 2010; Tesar et al., 2007). Based on the previous differences it has been proposed that there two pluripotent states, naive (ES cells) and primed (EpiSC) pluripotency (Nichols and Smith, 2009). Throughout this work the terms naive and primed pluripotency are used to refer to the potency of ES cells and EpiSC, respectively.

1.3.4 Embryonic germ cells

EG cells are pluripotent cells derived from PGCs (Brons et al., 2007; Evans, 1972; Evans and Kaufman, 1981; Martin, 1981; Matsui et al., 1992; Resnick et al., 1992; Tesar et al., 2007). PGC precursors are specified from the proximal posterior epiblast at day E6.25. The proximal epiblast acquires germ cell competence in response to morphogens produced by extra-embryonic tissues such as BMP4.

Therefore, it is likely that anterior-posterior axis specifying signals such as Nodal and Wnt antagonists play an important role in the specification of the germ cell lineage. In this regard, Smad2 mutants, which show an abnormal expression of Nodal and FGF8 throughout the epiblast present ectopic clusters of germ cells (Waldrip et al., 1998). PGCs can readily be identified from the proximal posterior epiblast by the expression alkaline phosphatase, Fragilis, Stella and Blimp1. Interestingly, the expression of pluripotency associated genes Sox2, Oct4 and Nanog is retained in PGCs. It is believed that the pluripotency associated factors act in cooperation with Blimp1 to suppress the activation of the somatic programme and erasure of epigenetic modifications. The established PGCs proliferate and migrate from the base of the allantois to reach the genital ridges at E11.5. During this period PGCs undergo a gradual erasure of the parental imprinting during their migration to the genital ridges, this is a critical step in restoring totipotency to the germline (Kato et al., 1999). Finally, once in the genital ridges, PGCs differentiate into germ cells.

Teratocarcinomas have been reported to spontaneously arise from germ cells (Stevens, 1960, 1964; Stevens and Little, 1954). However, the direct evidence of the pluripotent identity of germ cells remained anecdotal until the *in vitro* isolation of germ cells. In this regard, the cloning of LIF and steel growth factor proved to be pivotal for germ cell isolation (Matsui et al., 1991; Smith et al., 1988; Williams et al., 1988). Furthermore, it was also shown that supplementation of bFGF allowed for long term proliferation of germ cells (Matsui et al., 1992; Resnick et al., 1992).

1.3.5 Induced pluripotent stem cells

Takahashi and Yamanaka (2006) published a ground-breaking study showing that somatic cells could be induced to re-acquire an ES cell-like state. Indeed, the ectopic expression of Oct4, Sox2, cMyc and Klf4 and Sox2 is sufficient to induce pluripotency, albeit at low frequency and over a long time period, in terminally differentiated cell types. These reprogrammed cells are known as induced pluripotent stem (iPS) cells. Initially these cells were not able to generate germline competent adult chimaeras, this was only achieved once the reactivation of endogenous *Nanog* or *Oct4* were used to screen for successful reprogramming (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). Understanding the mechanism through which Oct4, Sox2, cMyc and Klf4 promote the induction of pluripotency is a key focal point for the iPS field.

1.3.5 Reprogramming of EpiSC

ES cells pluripotency can be restored in EpiSC by the overexpression of several ES cell transcription factors: Klf4, Klf2, Nr5a receptors, Nanog or activation of Jak/Stat3 activity (Guo and Smith, 2010; Guo et al., 2009; Hall et al., 2009; Silva et al., 2009; Yang et al., 2010). The overexpression of these factors alone is not sufficient to re-establish the ability to colonize pre-implantation embryos, but must be accompanied by a media switch. A pre-requisite for the induction of naïve pluripotency in EpiSC is the removal of the growth factors Activin and FGF (Hall et al., 2009), suggesting that in the majority of the cases, extrinsic cues have a dominant effect over intrinsic factors during EpiSC reprogramming. EpiSCs, co-cultured with fibroblasts, are susceptible to media-induce reversion into ES cell pluripotency. This

was achieved by the inhibition of mitogen-activated protein kinase (MEK; (PD0325901)) and/or glycogen synthase kinase 3 (GSK3; (CHIR99021)) signaling in EpiSC propagated on feeders (Greber et al., 2010).

LIF stimulation is crucial for the propagation of mouse ES cells (Smith and Hooper, 1987). EpiSC, on the other hand, respond poorly to LIF stimulation (Hall et al., 2009). This could be due partly, to the low levels of expression of components of the LIF/Stat3 signaling pathway or the absence of Stat3 partner proteins. In support of the first hypothesis, the ectopic expression of GY118F (chimeric receptor which induces the hyperactivation of JAK/Stat3) or Stat3 overexpression can revert EpiSC to ES pluripotency (Hall et al., 2009). This suggests that a competent LIF/Stat3 pathway is a limiting factor in the ability of cells to incorporate into pre-implantation embryos. Moreover, the overexpression of Klf4, a Stat3 target gene, can restore naïve pluripotency in EpiSC (Guo et al., 2009). Interestingly, EpiSC reprogramming efficiency is increased when Stat3 activation is combined with MEK (PD0325901)/GSK3 (CHIR99021) inhibition and ectopic Klf4 expression (Hall et al., 2009). The enhanced reprogramming efficiency of EpiSC observed by the ectopic expression of Klf4, in combination with activated Stat3, suggests that other Stat3 target genes may also contribute to the reprogramming process. Interestingly, another Klf family member, Klf2 has recently been shown to be able to revert EpiSC to naïve pluripotency. Unlike Klf4, Klf2 is not regulated by LIF-Stat3 signaling pathway. On the other hand, it has been demonstrated that Klf2 is a direct Oct4 target gene in ES cells (Hall et al., 2009).

Nr5a1 and Nr5a2 are orphan nuclear receptor whose function in pluripotent cells remains poorly understood. Nr5a2 has been reported to be able to replace Oct4 during somatic cell reprogramming (Heng et al., 2010). The reprogramming ability of the Nr5a receptor is intriguing, considering that these genes are not expressed in EpiSC and have no apparent role in ES cell self-renewal (Gu et al., 2005; Guo and Smith, 2010). It has been shown that Nr5a2 is necessary for the maintenance of Oct4 expression in the post-implantation epiblast (Gu et al., 2005). Moreover, it has been speculated that Nr5a2 also plays a role in the activation of Oct4 expression in ES cells (Masui et al., 2007). In a reprogramming context, Oct4 expression alone is insufficient to revert EpiSC to naïve pluripotency (Guo and Smith, 2010), this is an indication that the reprogramming activity of the Nr5a receptors acts also through an alternative mechanism.

Nanog has been reported to be essential for the acquisition of ground state pluripotency. Cells lacking Nanog can never acquire potency similar to that observed in the pre-implantation epiblast (Silva et al., 2009). In the context of somatic cell reprogramming through cell fusions, Nanog enhances reprogramming efficiency (Silva et al., 2006). Moreover, Nanog is also a potent inducer of ES cell pluripotency in EpiSC (Silva et al., 2009). Furthermore, Nanog is able to promote the reversion of EpiSC to naïve pluripotency in culture conditions that are not permissive for ES cell self-renewal (Theunissen et al., 2011).

1.4 Somatic Pluripotency

A defining feature of the amniote epiblast is its ability to produce all three embryonic germ layers and their differentiated derivatives, a property known as somatic pluripotency. The potency of a specific cell or tissue can be assayed by transplantation of the cell/tissue to ectopic sites in adult mice, such as the kidney capsule or testes, to test their differentiation potential. In this assay, a cell or tissue is pluripotent if it has the ability to generate teratocarcinomas. These tumours contain undifferentiated, pluripotent stem cells called EC cells and many differentiated tissues, including representatives of all three germ layers (Skreb et al., 1991). Using the teratocarcinoma assay it has been reported that somatic pluripotency in the mouse is lost at some point between the start of gastrulation (E7.5) and early organogenesis (E8.5) (Damjanov et al., 1971). After this time, embryonic cells are reported to be unable to form teratocarcinomas. It has also been shown that at E7.5 the different regions of the epiblast remain pluripotent (Beddington, 1983). Differentiation is thought to occur progressively in a rostral-to-caudal sequence. In part, this matches the expression pattern of the Oct4 (Yeom et al., 1996). Its disappearance in a rostral-to-caudal direction suggests that the primitive streak, at the posterior end of the embryo, retains pluripotent cells for longest. Heterotopic grafts of E7.5 epiblast have been reported to support this idea, as fragments of anterior epiblast transplanted to distal or posterior sites in the E7.5 embryo show slightly less capacity to adapt to their new location and differentiate appropriately than ectopically grafted distal or posterior epiblast (Beddington, 1982; Beddington, 1981). Although all grafts show a degree of both adaptation to their new environment and self-differentiation, these findings are by no means definitive in

support of the idea of a rostral-to-caudal sequence of commitment during gastrulation, up to E7.5.

Interestingly, somatic pluripotency is accompanied by the expression of key transcription factors known to regulate pluripotency in ES cells. These factors include Oct4 (Yeom et al., 1996), Sox2 (Avilion et al., 2003; Wood and Episkopou, 1999) and Nanog (Hart et al., 2004; Hatano et al., 2005). Sox2 expression is well documented and its expression continues long after E7.5 (Avilion et al., 2003; Wood and Episkopou, 1999), however, the expression of Oct4 and Nanog has not been monitored carefully over this period.

1.5 Factors governing self-renewal

Self-renewal in stem cells depends on extrinsic and intrinsic factors. The balance between extrinsic and intrinsic determines cell potency and fate. Intrinsic factors include cell autonomous components, whereas, extrinsic factors are external signals that control cell fate.

1.5.1 Intrinsic factors governing self-renewal

In the context of stem cell self-renewal, intrinsic regulators include cell autonomous components such as proteins involved in signaling transduction and nuclear factors controlling gene expression, protein modulation and chromatin modifications. Transcription factors can dramatically affect cell fate through the global regulation of gene expression, for this reason, these nuclear regulators receive particular attention. Transcription factors are traditionally defined as proteins that have the ability to bind to specific DNA sequences near the regulatory elements of

genes and promote transcription. In other words, these factors control gene expression, therefore affecting cellular function and fate. In general terms, transcription factors interact with each other at different levels and make up complex networks. The majority of these networks, including the pluripotency network, follow a hierarchical organization. This implies that the relative influence of each factor on the system is not identical. There are some factors that only affect a small number of network components, whereas other factors exert their influence on the majority of the elements that make up the network.

1.5.1.1 Oct4

Oct3/4 is POU containing transcription factor that is expressed in pluripotent populations and multipotent somatic progenitors (Nichols et al., 1998; Yeom et al., 1996). This homeodomain protein has been reported to be essential for the formation of the ICM (Nichols et al., 1998). It has been suggested that POU factors can interact with themselves forming homodimer or with other proteins to form heterodimers (Remenyi et al., 2003; Remenyi et al., 2001). Some of the proteins that are reported to interact with Oct4 are Sox2, β-catenin, Dax1 and Nanog (Ambrosetti et al., 1997; Ambrosetti et al., 2000; Sun et al., 2009; Takao et al., 2007; Wang et al., 2006). Additionally, Oct4 is a transcriptional regulator that can activate/repress target genes. Oct4 target genes in ES cells include: Oct4, Opn, FGF4, Utf1, Fbx15, Nanog, Zfp42 (Rex1), Esrrb, Lefty1, Cdx2 and Sox2 (Ben-Shushan et al., 1998; Botquin et al., 1998; Catena et al., 2004; Dailey et al., 1994; Nakatake et al., 2006; Nishimoto et al., 1999; Niwa et al., 2005; Tokuzawa et al., 2003; van den Berg et al., 2008; Wang et al., 2006; Zhang et al., 2008). Oct4 levels are critical for the specification of fate in mouse ES cells (Niwa et al., 2000). Normal diploid expression

of Oct4 is necessary to sustain ES cell self-renewal, however, a two fold increase in the levels of this transcription factor causes differentiation into primitive endoderm, while repression induces dedifferentiation into trophectoderm (Niwa et al., 2000). On the other hand, the interaction of Oct4 with Cdx2 has proven to be essential in the specification of cell fate in the blastocyst. Overexpression of Cdx2 in ES cells results in the repression of ES specific transcripts and differentiation into trophectoderm. Notably, Oct4 and Cdx2 interact at the protein and DNA level forming a reciprocal repression complex, in which the balance of Oct4/Cdx2 determines cell fate (Niwa et al., 2005).

1.5.1.2 Sox2

Sox2 belongs to the family of SRY (Sex-related HMG box) transcription factors that contain one HMG-box that binds DNA. Moreover, Sox2 interacts with Oct4 at the protein and DNA level (Ambrosetti et al., 1997; Ambrosetti et al., 2000). Interestingly, the majority of the pluripotency associated genes contain enhancers that possess Oct4/Sox2 binding motifs. Hints into the functionality of these enhancers are revealed by the fact that in undifferentiated ES cells they are highly active (Kuroda et al., 2005; Nakatake et al., 2006; Nishimoto et al., 1999; Tokuzawa et al., 2003; Yuan et al., 1995). Oct4 and Sox2 bind independently to their motifs, but synergistically activate the enhancers.

Sox2 deletion in the embryo is lethal at the post-implantation stage. In the absence of Sox2 the only surviving cells at this stage are giant trophoblast cells and extra-embryonic endoderm, however, this phenotype can be rescued by injection of wildtype ES cells (Avilion et al., 2003). This result suggests that Sox2 plays a critical role in the maintenance of the epiblast. In ES cells, Sox2 knockdown by siRNA

results in their differentiation into multiple lineages (Ivanova et al., 2006). However, a more rigorous analysis of Sox2 function in ES cells, using an inducible genetic, results in their differentiation into trophectoderm, reminiscent of the phenotype observed in Oct4 deletion. Notably, the phenotype observed following Sox2 could be rescued by expression of an Oct4 transgene (Masui et al., 2007). A more detailed analysis revealed that Sox2 is necessary in ES cells to sustain expression of Oct4 (Masui et al., 2007). The previous results suggest that Sox2 functions to stabilize Oct4 expression in ES cells.

1.5.1.3 Nanog

Nanog is a divergent homeodomain protein with the ability to confer cytokine-independent self-renewal to ES cells. It is first expressed in the early mouse embryo (morula, ICM and epiblast) and is permanently silenced from somatic cells at late head-fold stage, shortly before somitogenesis. It has been suggested that Nanog is necessary for the specification of naïve epiblast and germ cells. Recent studies imply that Nanog may have an essential role in the establishment of the pluripotent state, but is thereafter dispensable for its maintenance. ES cells that have been depleted of Nanog can self-renew indefinitely *in vitro* and retain pluripotency. It has been speculated that Nanog protein acts as a differentiation rheostat in pluripotent cells while improving their self-renewal ability (Mullin et al., 2008; Silva and Smith, 2008), however, the precise mechanism by which Nanog functions is poorly understood.

1.5.1.4 Esrrb

The estrogen related receptor-β (Esrrb) is an orphan nuclear receptor that has been reported to be an important component of the intricate pluripotency network (Chen et al., 2008; Ivanova et al., 2006; Kim et al., 2008; Loh et al., 2006; van den Berg et al.; van den Berg et al., 2008; Wang et al., 2006; Zhang et al., 2008). Previous studies have shown that pluripotency associated factors bind the regulatory region of the Esrrb gene (Chen et al., 2008; Ivanova et al., 2006; Kim et al., 2008; Loh et al., 2006; van den Berg et al.; van den Berg et al., 2008; Wang et al., 2006; Zhang et al., 2008). Nanog has been documented to interact with a range of partner proteins, among these interacting proteins is Esrrb (Wang et al., 2006; Zhang et al., 2008). Moreover, Esrrb has also been reported to interact at the protein level with Oct4, this interaction has been suggested to be important in the regulation of *Nanog* (van den Berg et al., 2008). Estrogen related receptor-β has also been shown to be essential in the self-renewal of mES cells (Ivanova et al., 2006; Loh et al., 2006) and also capable of somatic cell reprogramming when ectopically expressed in conjunction with Oct4 and Sox2 (Feng et al., 2009). Direct evidence for the role of Esrrb in ES cell self-renewal was obtained by the observation overexpression of this factor promotes cytokine independent self-renewal (Zhang et al., 2008). Nevertheless, the regulation of Esrrb in ES cells and the mechanisms through which Esrrb sustains pluripotency remain poorly understood.

1.5.1.5 Klf4

The Kruppel-like factor 4 (KLF4), also known as gut-enriched KLF, is a member of the Kruppel-like family of transcription factors. The Kruppel-like factors are zinc-finger proteins that share homology with the *Drosophila melanogaster*

segmentation gene, *Kruppel* (Schuh et al., 1986). Klf4 is expressed in ES cells and pre-implantation embryos (Guo et al., 2009; Li et al., 2005) and it has been shown to be a target of LIF/Stat3 signaling (Li et al., 2005; Niwa et al., 2009), suggesting that it may mediate the positive effects LIF stimulation. Direct evidence that it plays a role in ES cell self-renewal comes from the observation that Klf4 overexpression decreases differentiation in embryoid bodies (EB) and enhances the ability to generate secondary (Li et al., 2005). Notably, cytokine independent self-renewal can be achieved by Klf4 overexpression in ES cells (Hall et al., 2009). It has been reported that Klf4 cooperates with Oct4/Sox2 to activate Lefty1 expression (Nakatake et al., 2006). Moreover, comparative analysis of global gene expression following Oct4 deletions and Klf4 knockdown, showed a modest overlap between Oct4 and Klf4 target genes (Nakatake et al., 2006). Indeed, genome-wide chromatin immunoprecipitation (ChIP) analyses of ES cells revealed that Klf4 shares many common targets with the core pluripotency factors Nanog, Oct4 and Sox2 (Chen et al., 2008). Interestingly, it was shown that somatic cells could be reprogrammed to pluripotency by transfection with the transcription factors Oct4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006). Nonetheless, it was later demonstrated that cMyc and Klf4 are dispensable for the generation of iPS cells but their inclusion enhanced the speed and efficiency of the process (Nakagawa et al., 2008; Yu et al., 2007). Klf4 has also been shown to be capable of reverting EpiSCs to ES cell pluripotency (Guo et al., 2009). The previous observations provide further support to the notion that Klf4 plays a significant role in the regulation of naïve pluripotency. However, Klf4 knockdown in ES cells does not have a blatant phenotype, since alkaline-phosphatase immuno-reactivity and undifferentiated morphology remain unaffected (Jiang et al., 2008). Additionally, Klf4 deletion in

mice results in perinatal lethality due to the loss of skin barrier function and deficiencies in globlet cell differentiation (Katz et al., 2002; Segre et al., 1999). The lack of a blatant phenotype in Klf4$^{-/-}$ embryos and knockdown in ES cells may suggest that Klf transcription factors show functional redundancy. Consistent this notion, other members of the Klf family, such as Klf1, Klf2, and Klf5, can substitute for Klf4 in somatic cell reprogramming (Nakagawa et al., 2008). Furthermore, triple knockdown of Klf2, Klf4 and Klf5 results in ES cell differentiation, while single knockdowns did not exhibit blatant phenotypes (Jiang et al., 2008). Together, these results strongly suggest that Klf family members may play similar roles in the establishment and maintenance of naïve pluripotency. In summary, Klf4 forms part of the intricate network sustaining pluripotency, however, its precise role in ES cell self-renewal and maintenance of pluripotency remain poorly defined.

1.5.2 Extrinsic factors governing ES cell self-renewal

The original isolation of mouse ES cells was achieved using culture conditions used for routine propagation of EC cell lines. Such conditions involved the co-culture of pluripotent cells with inactivated fibroblast in serum-containing media (Evans and Kaufman, 1981; Martin, 1981). Given the observations that clonogenicity and self-renewal of pluripotent cells was enhanced under such conditions, it was believed that this effect was mediated by trophic factors secreted by fibroblasts. For this reason, inactivated fibroblasts are hence known as feeders. Further support for this notion was obtained by the observation that feeders could be replaced by the addition of medium conditioned by buffalo rat liver cells (Smith and Hooper, 1983). It was later shown that LIF was active component in the

conditioned medium (Smith et al., 1988; Williams et al., 1988). The combination of LIF and serum-containing media is sufficient to support self-renewal of undifferentiated cells. However, the active components present in serum remain allusive for quite some time. A ground-breaking study finally showed that bone morphogenetic protein 4 (BMP4) could replace serum, allowing for the first time the propagation of ES cells in fully defined conditions (Ying et al., 2003a). Given the observation that the propagation of pluripotent cells requires extrinsic signals, it is of particular interest to establish the link between intrinsic and extrinsic regulator mediating self-renewal in pluripotent cells.

1.5.2.1 LIF-Stat3

LIF belongs to the IL6 family of cytokines, all of which, signal through a common transmembrane receptor, gp130 (Boulton et al., 1994). In the presence of serum, stimulation of gp130 homodimers (Yoshida et al., 1994) or heterodimers (such as gp130 + LIF receptor) promotes ES cell self-renewal. Moreover, two main signaling pathways become activated following ligand-induced receptor dimerization. First, the intracellular domain of gp130 interacts with Janus kinases (JAKs) (Boulton et al., 1994) and subsequently, JAK phosphorylates the tyrosine residues on the intracellular domain of gp130. This phosphorylation is responsible for the generation of binding sites for STAT3. Once bound, STAT3 is phosphorylated on Tyr705 by JAKs, promoting subsequent dimerization and translocation to the nucleus where it directs transcription of target genes. Elimination of the tyrosine residues of gp130 essential for STAT3 binding abolished self-renewal activity (Niwa et al., 1998). Moreover, the overexpression of a fusion between STAT3-oestrogen Receptor (ER) promoted LIF independent self-renewal

when activated with exogenous tamoxifen (Matsuda et al., 1999). These experiments show that the critical target directing self-renewal through gp130 signaling is STAT3. Furthermore, STAT3 homodimers function as transcription factors. It is important to mention that the pluripotent epiblast forms normally in gp130$^{-/-}$ mutant embryos (Yoshida et al., 1996). Therefore, the *in vivo* requirement for gp130/STAT3 signaling in pluripotent cells remains unclear.

1.5.2.2 BMP4

Bone Morphogenetic Proteins (BMPs) are morphogens that play critical roles during embryonic development. In the context of ES cells, BMP4 has been shown to block neural differentiation (Ying et al., 2003a). Moreover, it was shown that BMP signaling acts through SMAD1, 5 and 8 to induce the expression of inhibitor of differentiation (ID) proteins. Confirmation that ID proteins mediate BMP function was obtained by the demonstration that constitutive expression of ID1, ID2 or ID3 can replace the requirement for BMP or serum for the propagation of ES cells (Ying et al., 2003a). It was also demonstrated that ES cells could be derived and propagated efficiently in chemically defined media (N2B27) supplemented exclusively with LIF and BMP4 (Ying et al., 2003a).

1.5.2.3 Wnt/GSK3

Wnt signaling has been reported to be involved in a vast number of functions in tissue homeostasis and development. There are two types of Wnt signaling pathways, canonical and non-canonical. Canonical Wnt signaling involves the presence of β-catenin, while non-canonical Wnt signaling operates without it. In canonical Wnt signaling, Wnt proteins bind the cell surface receptor Frizzled, such

an interaction leads to activation of Dishevelled. Subsequently, activated Dishevelled prevents the destruction complex from phosphorylating β-catenin, which is the signal necessary to target such protein for proteolysis. Therefore, inactivation of the destruction complex results in stabilization and nuclear accumulation of β-catenin. It has been shown that GSK3, a component of the destruction complex, is the kinase responsible for β-catenin phosphorylation. Furthermore, it was also demonstrated that direct inhibition of GSK3 could mimic Wnt signaling (Finlay et al., 2004). The role of Wnt signaling in ES cell self-renewal and differentiation has been a topic of debate due to contradictory findings (Del Valle et al., 2013; Haegele et al., 2003; Lyashenko et al., 2011; Otero et al., 2004; Wray et al., 2011). It has recently been demonstrated that canonical Wnt signaling is dispensable for ES cell self-renewal, but necessary for their differentiation into mesendodermal and neural lineages (Lyashenko et al., 2011; Wray et al., 2011). However, it has also been shown that GSK3 inhibition enhances ES cell self-renewal, through the modulation of Tcf3 (Wray et al., 2011). In vivo, β-catenin function is essential in anterior-posterior axis formation in the mouse (Huelsken et al., 2000).

1.5.2.4 ERK1/2

Mitogen activated protein kinases (MAPKs) have been implicated in the control of a great range of cellular processes. Two MAPKs, extracellular signal-regulated protein kinase-1 and -2 (ERK1/2), have been implicated in the regulation of early embryonic cell fate choice and ES cell self-renewal (Buehr and Smith, 2003; Burdon et al., 1999; Kunath et al., 2007; Lanner and Rossant, 2010; Saba-El-Leil et al., 2003; Stavridis et al., 2007). It should be noted that within the MAPK pathway,

MEK1/2 works upstream of ERK1/2. MAPKs are involved in the transduction of several extracellular cues, prominent among these, is the FGF signaling pathway. In brief, FGFs interact with its membrane bound receptors, which in turn induce the activation of the receptor tyrosine kinase, promoting the recruitment first of the SH2 domain containing proteins (Shp2/Grb2), and subsequently, son of sevenless homolog (SOS). This results in the induction of Ras, stimulating the MAPK cascade, which culminates with the activation of ERK1/2. It has been hypothesized that upon activation, ERK1/2 can act on cytoplasmic targets and/or translocate to the nucleus to activate transcription of its target genes (Marais et al., 1993; Yang et al., 2012).

Further evidence for the role of MEK in fate of mouse ES cells was obtained from the observation that during EB differentiation, MEK inhibition, prevents Oct4 downregulation (Burdon et al., 1999). Interestingly, FGF4$^{-/-}$ ES cells (Wilder et al., 1997) have been shown to display enhanced self-renewal. Furthermore, ES cells lacking functional *Fgf4* or *Erk2* failed to differentiate under conditions that normally promote neural differentiation, or in the presence of BMP4, which normally promotes non-neural differentiation (Kunath et al., 2007). The differentiation deficiency of Fgf4-null ES cell could be rescued by the addition of recombinant FGF4, demonstrating that the loss of autocrine FGF4 signaling is responsible for this phenotype (Kunath et al., 2007). Previous reports have shown that the activity of FGF/MAP kinase signaling pathway is essential for embryonic development, probably through its key role in the specification of early lineages in pre-implantation embryos (Chazaud et al., 2006; Feldman et al., 1995; Kang et al., 2013; Nichols et al., 2009; Yamanaka et al., 2010). *Fgf4*$^{-/-}$ embryos die shortly after

implantation (Feldman et al., 1995), prior to this, the ICM of these embryos were exclusively composed of Nanog-positive cells (Kang et al., 2013). Embryos lacking functional *Grb2* are unable to generate the primitive endoderm (PrE). Moreover, the ICM of Grb2-null embryos lack the mutually exclusive pattern of Gata6 and Nanog, which mark PrE and epiblast progenitors respectively, but instead display uniform Nanog expression (Chazaud et al., 2006). Interestingly, inhibition of FGF/MAP kinase signaling promotes epiblast formation, whereas excess FGF instructs the formation of primitive endoderm (Nichols et al., 2009; Yamanaka et al., 2010). The emerging view is that FGF signaling is critical for early lineage segregation in pre-implantation embryos and ES cells, probably through limited availability of soluble FGF and differential expression of FGF receptors in individual cells.

1.5.2.5 2i

It has recently been reported that ES cells can be efficiently propagated in well-defined media by the inhibition of differentiation inputs provided by ERK and GSK3 signaling (Ying et al., 2008). Thus, showing for the first time that, when shielded from differentiation cues, the pluripotency network can direct robust self-renewal in mouse ES cells. When cultured using the two inhibitors (2i), ES cells exhibit distinct gene expression and epigenetic features (Marks et al., 2012). A practical consequence is that it has become facile to establish ES cells from different strains of mice and also rats (Blair et al., 2011). It is noteworthy that, while the triple combination of 2i/LIF appears optimal, mouse ES cells can be propagated by providing any two of these three components (Wray et al., 2011), implying complementary inputs to a flexible gene regulatory network.

1.5.3 Extrinsic factors governing EpiSC cell self-renewal

1.5.3.1 TGFβ/Activin/Nodal

The transforming growth factor-β (TGFβ) signaling pathway is involved in numerous cellular processes in the developing embryo and adult organism. TGFβ superfamily of ligands includes: growth and differentiation factors, TGFβ, Activin, BMPs and anti-mullerian hormone. TGFβ super-family of receptors bind to its specific ligands, such interaction promotes the downstream signaling which include the phosphorylation of Smad2/3 (Shi and Massague, 2003). TGFβ signaling has been shown to be essential for the maintenance of pluripotency in hES cells and mouse EpiSCs (Amit et al., 2004; Brons et al., 2007; James et al., 2005; Tesar et al., 2007; Vallier et al., 2004). It has also been shown that MEF-conditioned medium can support the propagation of hES cells (Xu et al., 2001). A later study demonstrated that the active component promoting the propagation of mouse EpiSCs and hES cells cultured in MEF-conditioned medium was Activin (Greber et al., 2010). The previous finding is supported by reports showing that pharmacological inhibition of the TGFβ signaling pathway, results in the differentiation of mouse EpiSCs and hES cells (Greber et al., 2010; James et al., 2005; Wu et al., 2008). Recent studies have shown that TGFβ signaling acts through Smad2/3 to activate Nanog expression in mouse EpiSCs and hES cells (Greber et al., 2010; Vallier et al., 2009; Xu et al., 2008). Notably, long-term propagation of mouse EpiSC and hES cells requires supplementation with both Activin and FGF (Brons et al., 2007; Greber et al., 2010; Tesar et al., 2007; Vallier et al., 2004; Xu et al., 2005). The conserved role of TGFβ and FGF signaling across species (mouse, rat and human) is irrefutable evidence of the

evolutionary importance of these signaling pathways in the generation and propagation of pluripotent cells in mammals (Brons et al., 2007; Dvorak et al., 2005; James et al., 2005; Tesar et al., 2007; Vallier et al., 2004; Xu et al., 2005).

1.5.3.2 FGF

FGF2, also known as basic FGF, is now routinely added to the media for culture of hES cells and mouse EpiSC (Brons et al., 2007; Levenstein et al., 2006; Tesar et al., 2007; Xu et al., 2005). hES cells have been reported to be able to produce distinct isoforms of FGF2 and express several FGF receptor (Dvorak et al., 2005). Furthermore, inhibition of the FGF receptors in hES cells results in a reduction in phosphorylation of MEK1/2 and ERK1/2, followed by Oct4 downregulation and differentiation (Dvorak et al., 2005). Moreover, addition of recombinant FGF2 to cultures of hES cells does not seem to affect proliferation, but does reduce differentiation and the overall colony quality (Dvorak et al., 2005). Although this work provides evidence for the requirement of FGF signaling in the maintenance of pluripotency of hES cells, the mechanisms involved remain poorly defined. In this regard, it has been shown that FGF signalling has a moderate effect on *Nanog* expression in hES cells (Greber et al., 2007; Greber et al., 2010). Remarkably, in the mouse counterpart, EpiSCs, FGF signaling fails to support *Nanog* expression (Greber et al., 2010). Interestingly, the previous study also showed that FGF signaling stabilizes EpiSC pluripotency by blocking neuroectodermal differentiation and preventing de-differentiation into ES cells (Greber et al., 2010). It is quite intriguing that FGF signaling has such diverse roles in distinct pluripotent populations (hES cells; mouse ES cells and EpiSCs). However, it should be noted that FGF stimulation could induce multiple signaling cascades including MAPK-ERK, PLC-PKC and

PI3K-AKT. It is probable that any of these signaling pathways could affect the balance between self-renewal and differentiation of pluripotent cells. It is therefore necessary to dissect the role of these signaling cascades in order to identify their precise role in distinct pluripotent populations.

1.6 Cellular heterogeneity

Several studies have shown that under self-renewal conditions, mouse ES cells exhibit considerable heterogeneity in the levels of factors such as Nanog, Stella, Rex1, Pecam1, Tbx3 and Klf4 (Chambers et al., 2007; Furusawa et al., 2006; Hayashi et al., 2008; Toyooka et al., 2008). One could imagine that cellular heterogeneity reflects an irreversible developmental progression. However, sorting of single cells and monitoring their subsequent behaviour in culture have established that such heterogeneous states are reversible. For instance, it has been shown that within the undifferentiated sorted Nanog^{low} cells can give rise to a Nanog^{high} cells and *vice versa* (Chambers et al., 2007). Cellular heterogeneity could be advantageous during embryonic development as a mechanism that provides flexibility to the differentiation process. In this context, the balance of key transcription factors prompts differentiation along specific lineages when the correct cue arises. In ES cells low levels of Nanog result in pre-disposition towards differentiation, whereas high levels of Nanog present a bias towards self-renewal (Chambers et al., 2003; Chambers et al., 2007). Since Nanog is at the cusp of the pluripotency network and has the ability to modulate ES cell self-renewal, studying the origin of Nanog fluctuations is a key question to the field of pluripotent cell biology.

Chapter 2

Materials and Methods

2.1 Culture and manipulation of mouse ES cells

2.1.1 Routine culture

2.1.1.1 Media

For preparation of complete ES media, Glasgow Minimal Essential Media (GMEM; Sigma) was supplemented with 2-mercaptoethanol (BDH), sodium pyruvate/L-glutamine (Invitrogen), Foetal Calf Serum (FCS; Invitrogen), non-essential amino acids (Gibco) as indicated below. Leukemia Inhibitory Factor (LIF; in house) was added to 100U/ml.

GMEM/β-mercaptoethanol/10%FCS (hereafter referred to as GMEMβ/FCS):

- 500ml GMEM
- 11ml Sodium pyruvate (1mM)/L-glutamine (2mM)
- 51ml FCS
- 5.5ml Non-essential amino acids (100x)
- 555µl 2-mercaptoethanol (0.1 M)

2.1.1.2 Substrates

Gelatin (Sigma) was dissolved in Dulbecco PBS (Sigma) to a concentration of 0.1% w/v. Flasks/plates were covered with 0.1% gelatin in PBS for a minimum of 5-10 minutes at room temperature. Gelatin was aspirated and cells plated immediately.

Laminin was used as a substrate for ES cells that attached poorly in serum-free media. Briefly, flasks/plates were covered with poly-L-ornithine 0.01% (Sigma) and incubated at 4°C overnight. Poly-L-ornithine was aspirated and flasks/plates washed twice in PBS. Flasks/plates were then covered with laminin (Millipore) 5 mg/ml in PBS and incubated at room temperature for at least 2hr. Laminin was aspirated and replaced with media until ready to use.

2.1.1.3 Supplements and cytokines

Serum is the liquid fraction of clotted blood from fetal calf, depleted of cells, fibrin and clotting factors, but contains a large number of nutritional and macromolecular factors that promote survival and proliferation. Bovine serum albumin is the major component of the fetal bovine serum. FCS (Invitrogen) was diluted to a final concentration of 10% for routine culture of ES cells.

LIF was generated in-house by transient transfection of COS7 cells with plasmid encoding human LIF. Following 4 days, the conditioned media was harvested, titrated on ES cells and used at 100U/ml.

2.1.1.4 Reagents for passaging of ES cells

ES cells were passaged enzymatically using a 1x trypsin solution. The preparation was as follows, 0.186g of EDTA was dissolved in 500ml PBS and filter sterilised. Next, 5ml of trypsin (2.5%; Invitrogen) and chicken serum (Sigma) were added and subsequently mixed. The trypsin solution was stored at -20°C.

Protocol

ES cells were cultured as described in (Smith, 1991). Media was changed every day with pre-warmed GMEMβ/FCS/LIF. Cells were routinely passaged once they reached a confluency of 70-80% as follows:

1. Pre-treated tissue culture flasks/plates (Iwaki) were coated with 0.1% gelatin (Sigma)/Dulbecco PBS (Sigma) for 5-10 minutes before passaging cells.
2. ES cell media was removed from the flask/plates via aspiration.
3. Cells were washed once with pre-warmed Dulbecco PBS (Sigma), such solution was subsequently aspirated.
4. Trypsin solution was added to the cells. The volume of the trypsin solution varied depending on the type of flask/plate used. For maximal recovery cells it is essential that the monolayer be entirely covered with the trypsin solution.
5. The flask/plate was placed at 37°C/7% CO_2 incubator for ~3 minutes.
6. In order to dislodge the cells the flask/plate was gently tapped.
7. Trypsin was inactivated by addition of 4x volume of GMEMβ/FCS.
8. The cells were transferred to a centrifuge universal tube and centrifuged at 1200rpm for 3 minutes (ALC PK120; Annita).
9. The supernatant was aspirated and the cells pellet was resuspended in 10ml of GMEMβ/FCS/LIF.

10. Routinely the cells were split 1:10 at each passage into pre-warmed GMEMβ/FCS/LIF.
11. Cells were returned to the 37°C/7% CO_2 incubator. Flasks were left with a loose cap to ensure that the cells received CO_2.

2.1.2 Propagation in serum-free media supplemented with LIF and GSK3/MEK inhibitors

2.1.2.1 Media

Serum-free media, prepared as described (Ying et al., 2003b), is composed of a 1:1 mixture of Neurobasal (Gibco) and DMEM/F12 (Gibco), supplemented with 2-Mercaptoethanol (BDH), L-glutamine (Invitrogen), N2 (Gibco) and B27 (Gibco). The proportions and quantities mixed were as follows:

N2B27

- 1:1 Neurobasal:DMEM/F12
- 2mM L-glutamine
- 1:200 N2
- 1:100 B27
- 0.1 M 2-Mercaptoethanol

2.1.2.2 Inhibitors

Long-term propagation of ES cells in serum-free media was performed as described in (Ying et al., 2008) by supplementation of N2B27 with LIF and two small molecule inhibitors of GSK3 (CHIR99021) and MEK (PD0325901). Inhibition of

GSK3 was achieved using 3 μM CHIR99021 (Stemgent). MEK inhibition was achieved using 1μM PD0325901 (Stemgent).

2i/LIF See Ying et al., 2008

- N2B27
- LIF (100U/ml)
- CHIR99021 (3 μM)
- PD0325901 (1μM)

Protocol

ES cells were cultured in serum-free media supplemented with 2i/LIF as described (Ying et al., 2008). Media was changed every other day using pre-warmed N2B27/2i/LIF media. Cells were routinely passaged once that they reached a confluency of 70-80%.

1. Flasks/plates coated with gelatin or laminin as described in section 2.1.1.2.

2-8. These steps were identical to the protocol in section 2.1.1.4.

9. The supernatant was aspirated and the cells pellet was resuspended in N2B27/2i/LIF media and routinely split 1:10.
10. Cells were returned to the 37°C/7% CO_2 incubator. Flasks were left with a lose cap to ensure that the cells received CO_2.

2.1.3 Long-term storage of mouse ES cells

For long-term storage mouse ES cells can be frozen and kept in liquid nitrogen at a temperature of ~170°C. Cells were harvested by enzymatic dissociation (trypsin). A minimum of 2-3 million cells was resuspended in 1-0.5ml of freezing mix (GMEMβ/FCS/LIF containing 10% dimethyl-sulphoxide (DMSO)). The mixture was then transferred into a 1ml cryotube (Nunc). Vials were placed immediately at -80°C and after overnight storage transferred to liquid nitrogen.

2.1.4 Thawing mouse ES cells

Cryotubes were taken out of the liquid nitrogen storage (cell bank) and transferred immediately to the waterbath (37°C) to thaw. The thawed cells were then diluted with 9ml of pre-warmed GMEMβ/FCS/LIF and transferred into a centrifuge universal tube. The cells were pelleted via centrifugation at 1000rpm for 3-4 minutes (ALC PK120; Annita).

Table 2. 1 Antibiotics concentrations used for drug selection in mouse cells.

Antibiotics	Stock [ ]	Working [ ]	Supplier
Zeocin	100mg/ml	200μg/ml	Gibco
Blasticidin-S	5mg/ml	5-10μg/ml	Invitrogen
Hygromycin-B	50mg/ml	100-200μg/ml	Roche
G418	200mg/ml	200μg/ml	PAA
Puromycin	5mg/ml	1-2μg/ml	Sigma

2.1.5 Transfection of DNA into mouse ES cells

2.1.5.1 Stable transfection

ES cells at a density below 85% were used for stable transfections as follows:

1. Media was changed 2h prior to the electroporation.
2. 100mm diameter plates were gelatinized (see section 2.1.1.2).
3. 9ml of GMEMβ/FCS/LIF was added and the plates placed in a 37°C/7% CO_2 incubator to equilibrate.
4. ES cells were collected by enzymatic dissociation as described in section 2.1.1.4.
5. Cells were centrifuged at 1200rpm for 3 minutes (ALC PK120; Annita).
6. The cell pellet was washed twice with pre-warmed PBS.
7. Cells were resuspended in 10ml of PBS and counted using a haemocytometer.
8. 10^7 cells were centrifuged at 1200rpm for 3 minutes (ALC PK120; Annita).

9. The cell pellet was resuspended in a volume of 0.6ml PBS (1x).
10. 0.1ml of PBS (2x) was added to the 0.6ml of cell suspension.
11. 100µg of DNA (linearized with a restriction enzyme that cuts in the plasmid backbone) was resuspended in 0.1ml of PBS (1x) and transferred into an electroporation cuvette (Biorad).
12. The cell suspension was added to the electroporation cuvette (Biorad) containing the DNA and mixed gently.
13. The electroporation cuvette was left 3 minutes at room temperature.
14. Electroporation was performed at 0.8kV and 3µF using a GenePulser (Biorad).
15. The cell suspension was collected using a plugged Pasteur pipette and mixed with 9.2ml of pre-warmed GMEMβ/FCS/LIF.
16. 1ml of the cell suspension (10^6 cells) was added to the 100mm plates containing 9ml of pre-warmed and equilibrated GMEMβ/FCS/LIF.
17. The 100mm plates were swirled and placed in the 37°C/7% CO_2 incubator.
18. Antibiotic selection was added to the culture 24-30h post-transfection.

2.1.5.2 Episomal transfection

1. 10^6 cells were plated into a well of gelatinised 6-well plate in a volume of 2ml of GMEMβ/FCS/LIF.
2. The 6-well plate was placed in a 37°C/7% CO_2 incubator for 30 minutes prior to the transfection.
3. 3µl of Lipofectamine 2000 (Invitrogen) was diluted into 250µl of GMEMβ and incubated at room temperature for 5 minutes.

4. 3µg of plasmid DNA was diluted into 250µl of GMEMβ and incubated at room temperature for 5 minutes.
5. The Lipofectamine 2000 and DNA solutions were mixed and incubated at room temperature for 20 minutes.
6. The DNA/Lipofectamine mixture was added gently and slowly to the well with plated cells.
7. The 6-well plate was swirled to ensure proper mixing.
8. The 6-well plates were put back into the 37°C/7% CO_2 incubator.
9. The following day the transfected cells were replated and selection was started.

2.1.6 Picking ES cell colonies

ES cell colonies were picked once that the emerging colonies were visible with the naked eye. Media was then aspirated and cells washed twice with pre-warmed PBS. A very small volume of PBS was left on the 100mm dishes to ensure that the cells did not dry out. 30µl of trypsin was taken up using a 200µl pipette. The tip containing the 30µl of trypsin was carefully placed over the desired colony. The 30µl of trypsin was expelled over the colony. Next, the colony was scraped and sucked up with the 200µl pipette. The trypsinised cells were then seeded into a gelatinised well of a 96-well plate (Iwaki), which contained ~200µl of GMEMβ/FCS/LIF. The pipette was used for further mechanical dissociation of the colony before being discarded. The 96-well plates were returned to the 37°C/7% CO_2 incubator.

2.1.7 Clonal assays

2.1.7.1 Stable transfections

Clonal assays were routinely performed by seeding 600 cells into a gelatinised well of a 6-well plate. Cells were cultured in a 37°C/7% CO_2 incubator for 6 days in the presence or absence of LIF. After 6 days the cells were fixed and stained for alkaline phosphatase (see section 2.1.8).

2.1.7.2 Episomal supertransfections

Clonal assays were routinely performed by seeding at a density of 5 x 10^4 or 2 x 10^5 into a gelatinised 100mm plate. Cells were cultured in a 37°C/7% CO_2 incubator in the presence of the appropriate selection and with or without LIF for 10-12 days. After 10-12 days the cells were fixed and stained for alkaline phosphatase.

2.1.8 Alkaline phosphate staining

This was performed using an alkaline phosphatase kit (86R-1KT, Sigma).

2.1.8.1 Fixative solution

- 25ml Citrate solution (18mM Citric acid; 9mM Sodium citrate; 12mM NaCl)
- 8ml Formaldehyde
- 65ml Acetone

2.1.8.2 Stain

400µl FRV alkaline solution and 400µl Sodium nitrate solution were mixed and incubated for 2 minutes at room temperature. The Alkaline/Nitrate was added to 18ml dH_2O. Finally, 400µl of Napthol solution was added.

Protocol

1. Media was aspirated.
2. The cells were washed twice with pre-warmed PBS.
3. Fixative solution was added for 45s (with enough volume to cover the bottom of the well/plate).
4. The fixative solution was removed and the cells were washed with dH_2O.
5. The stain was added with enough volume to cover the bottom of the well/plate and incubated for 25 minutes at room temperature in the dark.
6. The stain was removed and wells were washed with dH_2O.
7. Plates were allowed to dry before subsequent analysis.

2.1.9 FACS analysis

ES cells were collected from the plates/flask using enzymatic dissociation (trypsin). The pellet was resuspended in ice-cold PBS/10% FCS at a density of ~10^6 cells. Anti-SSEA1 antibody (mc480IgM, Developmental Studies Hybridoma Bank, University of Iowa, U.S.A.) was added at a dilution of 1:1,000 and cells incubated at 4°C for 20 minutes. Next, cells were spun down and washed with PBS/10% FCS. Phycoerythrin-conjugated anti-IgM mouse antibody (Jackson Laboratories) was added at a dilution of 1:1,000 for 15 minutes at 4°C in the dark. To identify the dead cells, propidium iodide (Sigma) was used at a dilution of 1:5,000. FACS Calibur

(Becton Dickinson) and LSRFortessa (Becton Dickinson) were used to capture the data and Flowjo software was used for data analysis and presentation.

2.2 Derivation, culture and manipulation of mouse EpiSC

2.2.1 Routine culture of embryo-derived EpiSC

2.2.1.1 Media

Embryo-derived EpiSC were cultured as described (Tesar et al., 2007). Embryo-derived EpiSC media (DMEM-F12β/KOSR/Activin/bFGF) consisted of DMEM-F12 (Invitrogen), 20% Knockout Serum Replacement (KOSR; Invitrogen), 10 ng/ml basic FGF (bFGF; R&D Systems), 20 ng/ml Activin-A, 2 mM L-glutamine, 0.1 mM 2- mercaptoethanol, and 1x non-essential amino acids.

2.2.1.2 Substrate

Murine embryonic fibroblasts (MEFs) were obtained from E13.5 fetuses from 129/Ola mouse strain. Embryos were dissociated mechanically and by trypsinisation before plating on gelatin-coated 100mm diameter dishes in GMEMβ/FCS. The outgrowing cells were expanded for a single passage and used/frozen. For use as feeders, confluent MEFs were mitotically inactivated either by (1) exposure to 10μg/ml Mitomycin C (Sigma, M4287) for 3 hours or (2) irradiating at 30-60 gray. MEFs and feeders were maintained in GMEMβ/FCS.

2.2.2 Derivation of embryo-derived EpiSC

EpiSC were derived from embryos obtained from different mouse stains such as 129/Ola, C57BL/6, 129/*Nanog*:GFP or B6/129/Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$/Col1a1$^{tm2(tetO-Pou5f1)Jae/J}$.

Protocol

1. Embryos were dissected from their decidua in M2 or PB1.
2. Reichert's membrane was removed with forceps or glass needles and a cut was made at the boundary between the extra-embryonic ectoderm and the epiblast with forceps.
3. The embryonic fragment containing the epiblast and overlying visceral endoderm was incubated for 10-15 minutes at 4 °C in a solution of 0.5% trypsin and 2.5% pancreatin in PBS.
4. Embryos were drawn into a hand-pulled glass pipette with a diameter slightly smaller than the fragment to peel away the visceral endoderm.
5. The isolated epiblast fragments were rinsed in PB1 and disaggregated into clumps with a very fine glass pipette. For the derivation of anterior/posterior EpiSC lines from E7.5-E7.75 each embryo was carefully dissected as described in section 2.3.4. Briefly, the anterior/posterior epiblast and overlying visceral endoderm was incubated for 10-15 minutes at 4°C in a solution of 0.5% trypsin and 2.5% pancreatin in PBS. The epiblast fragments and visceral endoderm were rinsed in PB1 and disaggregated into clumps with a very fine glass pipette.

6. The embryo fragments were then explanted onto a layer of feeders in wells with an area of 1.9 cm^2. Primary explants and EpiSC were cultured on feeders in DMEM-F12β/KOSR/Activin/bFGF.
7. Feeders were seeded at a density of 4.8 x 10^4 cells per cm^2 in GMEMβ/FCS.
8. Primary explants were passaged every 2–3 days using Accutase. EpiSC were passaged every 5-6 days with Accutase.
9. Cells were incubated with Accutase for 5 minutes and then triturated into small clumps of 10–100 cells.
10. Cells were then resuspended in DMEM-F12β/KOSR/Activin/bFGF and replated on a layer of feeders in the appropriate culture plates.

2.2.3 Routine culture of ES-derived EpiSC

2.2.3.1 Media

ES-derived EpiSC were cultured as described previously (Guo et al., 2009) in media consisting of N2B27 supplemented with Activin-A (20 ng/ml) and bFGF (10 ng/ml).

2.2.3.2 Substrate

Human fibronectin (Sigma) was dissolved in Dulbecco PBS (Sigma) to a concentration of 15 μg/ml. Flasks/plates were covered with 15 μg/ml human fibronectin in PBS for a minimum of 5-10 minutes at room temperature. Fibronectin was aspirated and cells plated immediately.

2.2.4 Derivation of ES-derived EpiSC

ES-derived EpiSC were generated as described (Guo et al., 2009).

Protocol

1. ES cells were plated at a density of 4 x 10^4 in a 35mm diameter dish (6-well plates, Iwaki) with fibronectin.
2. For the first 24hrs the cells were grown in GMEMβ/FCS and then transferred to feeder free EpiSC media (N2B27/Activin/bFGF).
3. Cells were passaged every 5-7 days; briefly, cells were washed twice with PBS and then incubated in 350µl of Accutase (Sigma) for 3-5 minutes.
4. 3ml of N2B27 was used to dilute the Accutase and collect cells.
5. Cell suspension was then placed in a universal and spun down for 3 minutes at 1400rpm.
6. EpiSC were replated at a density of 4 x 10^4 in N2B27/Activin/bFGF.

2.2.5 Long-term storage of EpiSCs

For long-term storage mouse EpiSCs were frozen and kept in liquid nitrogen at a temperature of ~170°C. Cells were harvested by enzymatic dissociation (Accutase). A minimum of 2-3 x 10^6 cells was resuspended in 1-0.5ml of KOSR with 10% DMSO. The mixture was then transferred into a 1ml cryotube (Nunc). Vials were placed immediately at -80°C and after overnight storage transferred to liquid nitrogen.

2.2.6 Thawing mouse EpiSCs

Cryotubes were taken out of the liquid nitrogen storage (cell bank) and transferred immediately to the waterbath (37°C) to thaw. The thawed cells were then diluted with 4ml of pre-warmed N2B27 media and transferred into a centrifuge universal tube. The cells were pelleted via centrifugation at 1000rpm for 3-4 minutes (ALC PK120; Annita).

2.2.7 Differentiation of EpiSC using signal inhibition

EpiSCs were plated at a density of 1 x10^4. FGF and TGFβ/Nodal/Activin signaling pathway were inhibited using PD0325901 (1μM; Stemgent) or SB431542 (10μM; Sigma) respectively.

2.2.8 Reprogramming of EpiSC

2.2.8.1 Episomal supertransfection

EpiSC expressing polyoma large T antigen were transfected with 3μg of plasmid using Lipofectamine 2000 (Invitrogen; 11668-019). Next day, 5 x 10^4 cells were replated in the presence of the appropriate drug selection and plates stained for AP after 7 days. For further analysis, Epi-iPS cell colonies were picked and expanded in the absence of selection.

2.2.8.2 Stable transfection

For stable integration of plasmid DNA into EpiSC, Amaxa Nucleofection (VPH-1001; Lonza) was used. Reprogramming of EpiSC with stably integrated

plasmid DNA was ideally performed with a Cre-revertable plasmid. To establish Cre-revertable transgenic EpiSC lines, 5 x 10^6 RC EpiSC were nucleofected using the Amaxa Nuclefection kit (VPH-1001; Lonza) with 10μg of the desired vectors. Transfected cells were plated in the appropriate EpiSC culture media and placed under antibiotic selection for a period of 5 days. Following selection, cells were replated at a density of 2 x 10^5 into N2B27/2i/LIF in continued drug selection and plates stained for alkaline phosphatase after 7 days. For further analysis of reverted clones, Epi-iPS cell colonies were picked and expanded in GMEMβ/FCS/LIF. To test clone stability, Cre-mediated excision of the plasmid DNA was performed.

2.2.8.3 Reversion of EpiSC to Epi-iPS in a *Nanog*$^{-/-}$ background

5 x 10^4 EpiΔN-iNanog and EpiΔN-iEsrrb cells were replated in 100mm diameter dishes in GMEMβ/FCS/LIF +/- doxycycline and plates stained for AP after 7 days. Epi-iPS cell colonies were also picked and expanded in the absence of doxycycline.

2.3 Mouse husbandry, staging, culture and manipulation of embryos

2.3.1 Mouse husbandry and staging

Mouse husbandry was performed by the animal unit staff at the ISCR/SCRM. Mice were maintained on a 12 hour light/12 hour dark cycle. For timed mating's, noon on the day of finding a vaginal plug was designated E0.5. Except where stated, wild-type embryos were MF1 or 129/Ola strain. Due to

embryonic stage variation between and within litters, gastrulation-stage embryos were more carefully staged according to (Downs and Davies, 1993), namely: ES, early-streak; MS, mid-streak; LS, late-streak; OB, no allantoic bud; EB, early allantoic bud; LB, late allantoic bud; EHF, early head fold; LHF, late head fold.

2.3.2 Blastocyst injections and morula aggregations

Chimaeras were produced by microinjection into C57Bl/6 blastocysts or by morula aggregation as described (Schwartzberg et al., 1989).

2.3.3 Embryo culture

Embryo culture was performed in 50% rat serum as described (Copp, 1990), except that embryos were cultured in static 4-well dishes in an incubator at 5% CO_2 in air.

2.3.4 Embryo manipulation (mechanical)

Embryos were dissected in M2 (Sigma) using a dissecting microscope (Olympus SZ40). Sub-regions of the embryo were separated with hand-pulled solid glass needles as described (Cambray and Wilson, 2007). To dissect anterior (*Nanog*:GFP negative) and posterior (*Nanog*:GFP positive) regions of the E7.5 embryo, embryos were first ordered relative to developmental stage and photographed in a fluorescence stereomicroscope to assess GFP positive and negative regions. A transverse cut was made to separate the most proximal epiblast, containing primordial germ cells, together with the extra-embryonic region, from the distal epiblast. A second oblique cut was made to separate the distal/anterior

from posterior/proximal regions. Finally, the embryos were again viewed and photographed to check accuracy of micro-dissection. Where a minority of GFP positive cells remained in anterior regions, these were trimmed off and discarded.

To dissect prospective forebrain and adjacent region, the extra-embryonic membranes were removed with forceps and the embryo lay flat dorso-ventrally, with the aid of a transverse cut near the node if necessary. Forebrain and adjacent region were then cut with glass needles.

2.3.5 Embryo manipulation (biological)

Ubiquitous Oct4 expression in somitogenesis-stage tissue was achieved using a mouse line carrying both the Doxycycline-inducible reverse tetracycline transactivator (rtTA) targeted at the *Col1a1* locus, and an Oct4 transgene (*TgOct4)* under the control of a tet-response element, targeted at the Rosa26 locus. 129/Ola wild type females were crossed with *TgOct4/TgOct4;rtTA/rtTA* homozygous male mice (B6/129/Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$/ Col1a1$^{tm2(tetO-Pou5f1)Jae/J}$; (Hochedlinger et al., 2005).

2.3.6 Grafting under the kidney capsule

Engraftments were performed as described by Robertson (1987), with the following modifications. Surgical operations were carried out on mice of the same strain as the fragments grafted. Grafting was done in a Class II laminar flow hood under sterile conditions using a dissecting microscope (Olympus SZ40). Mice were anaesthetised with an intra-peritoneal injection of 0.5 ml of Avertin. To reduce the inflammation due to procedure, 0.1ml 1% Turbogesic was also injected intra-

peritoneal. An incision was made in the flank on the left side and the corresponding kidney exposed. A small tear was made in the kidney capsule using fine forceps and fragments were transferred using a glass pipette controlled by mouth suction. The kidney was then replaced, the peritoneal cavity sutured and the skin clipped to close the wound.

2.3.7 Recovery and processing of kidneys

Kidneys were processed, fixed and stained as described (Bancroft & Gamble, 2002). 4-6 weeks after the graft was performed, the mice were sacrificed and the kidneys removed in PBS. Growths could be seen by eye or in some cases a dissecting microscope was used. After imaging, the growth and the surrounding part of the kidney were fixed overnight in 4% PFA/PBS. After fixing, the kidneys were dehydrated through ethanol series (2x 5min PBT; 1x 5min 25% methanol/PBT; 1x 5min 50% methanol/PBT; 1x 5min 75% methanol/PBT; 2x 5min 100% methanol), cleared in xylene and embedded in paraffin wax before being sectioned in a microtome. Alternatively, for antibody and his to-chemical stains, the tissue was sectioned in a Leica cryostat at 5µm.

2.3.8 Isolation of secondary EpiSC from teratocarcinomas

Tumours were isolated and chopped roughly before dissociation in 0.5% trypsin/2.5% pancreatin/PBS and incubation at 37°C for 15 minutes. After trituration, cells were plated onto MEFs in EpiSC media, passaged the following day and subsequently every 2-3 days.

2.4 Imaging, staining and in situ hybridisation

2.4.1 Imaging

Images were captured using Volocity (Improvision) software on a Zeiss Stemi SV11 dissecting microscope (whole embryos and kidneys), an Olympus IX51 (for cultured cells), or an Olympus BX61 (for sections). Image processing was performed using Adobe Photoshop software.

2.4.2 Immunohistochemistry of embryos

Embryos were dissected in PB1 or M2, the extra-embryonic membranes were removed to facilitate penetration of the antibodies.

Protocol

1. The embryos were fixed using 4% PFA/PBS overnight at 4°C.
2. Permeabilization was achieved using 0.5% Triton X100/PBS for 15 minutes.
3. Blocking was done overnight at 4°C using 3% serum (same species as secondary antibody), 1% BSA/PBS/Triton (0.1%).
4. The primary antibody was diluted appropriately in blocking solution and the embryos were incubated with the diluted antibody for 48hrs at 4°C.
5. The embryos were then washed four times with PBS/Triton (0.1%) for 15 minutes at RT.
6. The secondary antibodies conjugated to Alexa fluorophores (Molecular Probes, Eugene, Oregon, United States) were diluted 1:1000 in blocking buffer and the embryos were incubated with the diluted antibody for 3hrs in the dark at RT.

7. Embryos were then washed four times with PBS-Triton (0.1%) for 15 minutes at RT.

2.4.3 Immunohistochemistry of cells

Protocol

1. Cells were fixed in 4% PFA/PBS for 10 minutes at RT.
2. Permeabilization was done with PBS-Triton (0.5%) for 15 minutes at RT.
3. Blocking was performed for 30 minutes at RT using PBST 0.1%/BSA 1%/ Serum 3%.
4. Primary antibodies were diluted in blocking buffer and applied for 1-2 h at RT or overnight at 4 °C.
5. Cells were washed three times in PBS/Triton (0.1%).
6. Secondary antibodies were diluted 1:1000 in blocking buffer and applied for 1 h at RT.
7. The cells were washed at least three times in PBS/Triton (0.1%).

The cells and embryos were then visualized on an Olympus inverted fluorescence microscope. Nanog was detected by using an affinity-purified rabbit anti-Nanog antibody directed against an amino-terminal epitope (SVGLPGPHSLPSSEE; Chambers, 2005), 1:400; Oct4 (sc-5279, Santa Cruz Biotechnology), 1:200; Sox2 (sc-17320, Santa Cruz Biotechnology), 1:200; Esrrb (PP-H6705-00, Persaeus Proteomics), 1:1000; Hnf3B (Foxa2; sc-9187, Santa Cruz Biotechnology), 1:200; T-Brachyury (sc-17745, Santa Cruz Biotechnology), 1:400; SSEA-1(MC-480) (ab16285 , Abcam), 1:100.

For immunostaining of tumours, paraffin sections were re-hydrated and after 15 minutes of antigen retrieval by heating and blocking, sections were incubated with anti-Oct4 antibody (1.5 µg/ml, sc-5279, Santa Cruz Biotechnology), anti-Tubulin III (Covance; 1/300 dilution) or anti-desmin (D33, DakoCytomation, 1/50 dilution) overnight. Sections were then washed three times in PBS/0.1%v/v TritonX100 and incubated with a secondary biotinylated goat anti-mouse antibody (7.5 µg/ml, BA-9200, Vector Laboratories). After 30 minutes of quenching of endogenous peroxidise activity using 0.3% H_2O_2 DAB detection was carried out using the Avidin Biotin Complex (ABC) reagent kit (Vector Laboratories) according to the manufacturer's instructions.

2.4.4 In situ hybridization

Embryos were subjected to whole-mount in situ hybridisation as described (Wilkinson et al., 1990) except that proteinase K treatment was empirically adjusted between 8-16 minutes according to embryo size and stage. The riboprobes used were: *Oct4* (Scholer et al., 1990a) and *Nanog* (Chambers et al., 2003). The T7 or Sp6 polymerase site was used to transcribe antisense riboprobe. Embryos were dehydrated via an ethanol series (2x 5min PBT; 1x 5min 25% methanol/PBT; 1x 5min 50% methanol/PBT; 1x 5min 75% methanol/PBT; 2x 5min 100% methanol), processed for paraffin wax histology and sectioned in a microtome.

2.5 Molecular biology

2.5.1 RNA, protein and DNA extraction

2.5.1.1 RNA extraction

Total RNA from cultured cells was isolated using the RNeasy microkit or minikit (Qiagen), and performing on-column digestion with DNase I (Qiagen).

2.5.1.2 RNA isolation with Trizol

Protocol

1. Lyse cells by adding 1ml of Trizol. Make sure to pipette the cell lysate several times to ensure proper homogenization.
2. Incubate the homogenized samples for 5min at 15-30°C to allow for complete dissociation of nucleoprotein complexes.
3. Add 0.2ml of chloroform per 1ml of Trizol used. Shake simples vigorously by hand for 15sec and incubate them at 15-30°C for 2-3min.
4. Centrifuge the samples at no more than 12,000g for 15min at 2-8°C (ALC PK120; Annita).
5. Phase separation should be visible: (i) colourless upper aqueous phase (RNA), interphase (DNA) and lower phenol-chloroform phase (proteins and some DNA).
6. Transfer the aqueous phase to a fresh tube.
7. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol in a ratio of 1:1. Incubate samples at 15-30°C for 10min and

centrifuge at no more than 12,000g for 10min at 2-8°C (ALC PK120; Annita). The RNA is in the pellet.

8. Remove the supernatant and wash the RNA pellet with 500µl of 75% Ethanol. Mix the sample by vortexing and centrifuge at no more than 7,500g for 5min at 2-8°C (ALC PK120; Annita).
9. Resuspend the RNA pellet in 87µl of RNAse free H_20 and incubate at 56°C for 10min.
10. Add 3µl of DNase I and 10µl of RDD buffer. Incubate 10min at RT.
11. Inactivate the DNAse I by incubating at 75°C for 15min.
12. Add 50µl of phenol (pH 4.2) and 50µl of chloroform. Vortex the sample.
13. Centrifuge the samples at no more than 12,000g for 15min at 2-8°C (ALC PK120; Annita).
14. Transfer the aqueous phase to a fresh tube with 10µl NaOAc 3M pH 5,2.
15. Add 300µl of 100% ethanol. Incubate 2-4hrs at -80°C or overnight at -20°C.
16. Centrifuge the samples at no more than 12,000g for 10min at 2-8°C (ALC PK120; Annita).
17. Wash the RNA with 75% ethanol.
18. Dissolve the RNA in 50µl of RNAse free H_20. Incubate at 56°C for 10min.

2.5.1.3 First strand cDNA synthesis

Reverse transcription reactions were performed on 30 ng-2ug of total RNA in a final volume of 20µl with 100U of SuperScriptIII (Invitrogen) and 200ng random hexamers (Invitrogen) or 250ng of oligodT_{12-18} (Invitrogen) primers at 42°C for 60 minutes.

2.5.1.4 Real-time PCR

Real-time RT–PCR reactions were performed in triplicate in 384-wells plates with a 480 LightCycler (Roche) using LightCycler 480 SYBR Green I Master (Roche). 5µl of cDNA or immuno-precipitated chromatin were used per reaction. Standard curves of all primers were performed to check for efficient amplification, and all melting curves were generated to verify production of single DNA species with each primer pair. Values for each gene were normalised to expression of TATA box Binding Protein (TBP).

Table 2. 2 Primers used for qPCR

Target	Sequence
TBP (sense)	GGG GAG CTG TGA TGT GAA GT
TBP (antisense)	CCA GGA AAT AAT TCT GGC TCA
Oct4 (sense)	GTT GGA GAA GGT GGA ACC AA
Oct4 (antisense)	CTC CTT CTG CAG GGC TTT C
Rex1 (sense)	CAG CTC CTG CAC ACA GAA GA
Rex1 (antisense)	ACT GAT CCG CAA ACA CCT G
FGF4 (sense)	CCG GTT CTT CGT GGC TAT GA
FGF4 (antisense)	CTT ACT GAG GGC CAT GAA CAT
Brachyury (sense)	CAG CCC ACC TAC TGG CTC TA
Brachyury (antisense)	GAG CCT GGG GTG ATG GTA
KLF4 (sense)	GGC GAG AAA CCT TAC CAC TGT
KLF4 (antisense)	TAC TGA ACT CTC TCT CCT GGC A
Sox2 (sense)	GGC GGC AAC CAG AAG AAC AG
Sox2 (antisense)	GCT TGG CCT CGT CGA TGA AC
FGF5 (sense)	TTG CGA CCC AGG AGC TTA AT
FGF5 (antisense)	CTA CGC CTC TTT ATT GCA GC
Hnf3B (Foxa2; sense)	CAT CCG ACT GGA GCA GCT A
Hnf3B (Foxa2; antisense)	GCG CCC ACA TAG GAT GAC
Esrrb (sense)	TGG CAG GCA AGG ATG ACA GA
Esrrb (antisense)	TTT ACA TGA GGG CCG TGG GA
Dppa3 (Stella; sense)	GAT GCA CAA CGA TCC AGA TTT
Dppa3 (Stella; antisense)	TGG AAA TTA GAA CGT ACA TAC TCC AA
KLF2 (sense)	CTA AAG GCG CAT CTG CGT A
KLF2 (antisense)	TAG TGG CGG GTA AGC TCG T

2.5.1.5 Plasmid preparation from bacteria

Overnight culture of DH5α bacterial cells containing the desired plasmid was performed in the appropriate drug selection. Next day, the plasmid DNA was isolated using Mini, Midi or Maxiprep kits (Qiagen). The final plasmid concentration was determined using a ND-1000 spectrophotometer.

2.5.1.6 Ethanol precipitation

Protocol

1. Adjust pH of sample by adding sodium acetate (3M) to a final concentration of 0.2M.
2. Add 2.5 volumes of ice cold ethanol (100%).
3. Vortex the sample and then place at -20°C for 30min.
4. Microcentrifuge at max speed at 4°C for 10min.
5. Aspirate the ethanol.
6. Wash pellet with 500µl of ice cold ethanol (70%).
7. Microcentrifuge at max speed at 4°C for 5min.
8. Aspirate the ethanol.
9. Allow pellet to air dry for 5 min.
10. Resuspend pellet in the desired volume of H_20 or PBS.

2.5.2 DNA manipulation

2.5.2.1 Restriction endonuclease digestion

Plasmid DNA was digested enzymatically using restriction endonucleases from Roche and NEB. Enzymatic digestion was performed according to the manufacturer's instructions.

2.5.2.2 Ligation

DNA ligations were performed using either T4 DNA ligase or Quick T4 DNA Ligase (NEB). Ligations were set up with a 1:3 molar ratio excess of insert, using 50ng of vector. Ligations reactions were set up according to the manufacturer's instructions.

2.5.2.3 Agarose gel electrophoresis

Preparation of 1% TBE gel

200ml of 0.5x TBE buffer (1mM EDTA + 45mM Tris-Borate) was mixed with 2g of agarose (Cambrex). The mixture was heated in a microwave until the agarose was completely dissolved. 6µl of ethidium bromide (10mg/ml) was added when the agarose had cooled below 60^{o}C and gels were poured into casting trays. Electrophoresis was performed in Mupid gel tanks at 25-130V in 0.5x TBE buffer. 1x DNA loading buffer with Ficoll (6x stock; Sigma) was used to label the DNA. Nucleic acid was visualized by UV trans-illumination (GeneFlash Imager; Syngene).

2.5.2.4 Purification of digested DNA fragments

Bands were purified from agarose gels by excision of the desired size using a clean scalpel blade on a trans-illuminator. Qiagen Gel Extraction Kit (Qiagen, 28704) was used for purification of nucleic acids according to the manufacturer's instructions.

2.5.2.5 Transformation of plasmid DNA into bacteria

Luria Broth (LB) agar

- Yeast extract (5%; Difco)
- NaCl (5mM)
- Agar (1.5%)
- Tryptone (pancreatic digest of casein; 1%; Difco)

Protocol

1. LB agar was melted, cooled to below 60^{o}C and appropriate antibiotics added.
2. LB agar (+antibiotics) was poured into plates.
3. 50µL of competent DH5α (*E. coli*; Invitrogen) were thawed on ice.
4. 10ng of plasmid DNA was then transformed into competent DH5α and incubated in ice for 30 minutes.
5. The bacterial cells were then heat shocked for 30 seconds at 37C and then returned onto ice for 2 minutes.
6. 950µl of LB added and tubes shaken at 37^{o}C for 1 hour on an orbital shaker.
7. 10 or 100µl of transformed bacterial cells were plated on the appropriate selective plates.

8. Plates were incubated overnight at 37ºC.

2.5.2.6 Topo-cloning

Cloning of blunt end PCR products were performed using the Zero Blunt® TOPO® cloning kit (Invitrogen). TOPO® TA Cloning Kit (Invitrogen) was used for PCR products with a polyA overhang. Reactions were carried out according to the manufacturer's instructions. Colonies formed on ampicillin plates were mini-prepped and test digested with EcoR1 to check for insertion of the PCR product. PCR products were subsequently verified by DNA sequencing (School of Biological Sciences Sequencing Service, University of Edinburgh).

2.5.3 Western Blotting

2.5.3.1 Solutions and reagents

Laemmli Lysis Buffer:	2% SDS, 10% Glycerol, 60mM Tris pH6.8, 0.1M DTT, Bromophenol blue
Running Buffer:	NuPage MOPS, 20x (Invitrogen)
Antioxidant:	NuPage Antioxidant (Invitrogen)
Transfer Buffer:	20% MeOH, 25mM TRIS, 192mM Glycine, in dH2O
TBS:	10mM TRIS, 150mM NaCl, In dH2O, pH7.6-8.0

Blocking Buffer:	10% dried, skimmed milk (Marvel), 0.05% NP40, in TBS
Incubation Buffer:	5% dried, skimmed milk (Marvel), 0.15% NP40, in TBS
Wash Buffer:	10mM TRIS, 650mM NaCl, 0.3% Triton x100, in dH2O, pH7.6-8.0
Stripping Buffer:	2% SDS, 62.5mM TRIS, pH6.8
Gels:	10% NuPage Bis-Tris (Invitrogen) 12-14% NuPage Bis-Tris (Invitrogen)
MW marker:	SeeBlue Plus2 (Invitrogen)
Gel-tank:	Novex Mini-Cell (Invitrogen)
Membrane:	Nitrocellulose (Hybond-ECL, Amersham)
ECL:	ECL-plus (Amersham)

2.5.3.2 Sample Preparation

~2 x 10^6 cells were lysed directly on the wells of 6-well plates in 250µL laemmli lysis buffer and transferred immediately to eppendorf tubes on ice. The

samples were boiled for 5 minutes, sonicated briefly and subjected to SDS-PAGE directly or stored at -20C.

2.5.3.3 Running the Gel

10% or 12-14% Novex gels (Invitrogen) were used. The comb was removed, wells rinsed by pipetting running buffer into the wells and 20µL sample loaded per well. 10µL protein standard was loaded in 1 well. Gels were run at 200V for approximately 1hr.

2.5.3.4 Transfer

The gel was removed from its casing into transfer buffer, the foot and wells removed, and assembled with the membrane between 1 sponge and 2 sheets wet Wattman paper either side, taking care to avoid bubbles. The assembly was transferred at 395 amps for 70 minutes in a cold room in BioRad gel tanks.

2.5.3.5 Blocking, antibody incubation, washing and stripping

The membrane was placed protein side up in a 50ml tube and incubated for 2hrs in blocking solution. Primary antibodies were diluted in 5ml (PBT/10% milk) and incubated with the membrane for 2hrs at RT or overnight at 4°C. Secondary antibodies were diluted in PBT/10% milk and incubated with the membrane for 1hr at RT. Following primary and secondary antibody incubations membranes were washed 3 x 15 minutes in 30ml wash buffer. Membranes were incubated for 5 minutes with ECL, exposed to X-ray film and developed. If membranes were to be re-probed antibodies were stripped by incubating for 30 minutes at 70°C in stripping buffer. Following stripping membranes were re-blocked.

Table 2. 3 Antibodies used for western blot

Antibody	Supplier	Dilution	2º antibody
Nanog	**Chambers, 2005**	**1:2000**	**Rabbit-HRP**
Oct4 (C10)	**Santa Cruz**	**1:1000**	**Mouse-HRP**
Sox2 (Y17)	**Santa Cruz**	**1:1000**	**Goat-HRP**
Esrrb	**Persaeus Proteomics**	**1:1000**	**Mouse-HRP**
HDAC	**Upstate**	**1:1000**	**Mouse-HRP**

2.7 Bisulphite genomic sequencing

DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen) and bisulphite-converted using the Imprint DNA modification kit (Sigma). The treated DNA was then PCR amplified using Platinum Taq DNA polymerase (Invitrogen) and the following *Nanog* promoter-specific primers: MeNanog-F2-S, GAT TTT GTA GGT GGG ATT AAT TGT GAA TTT and MeNanog-F2-AS, ACC AAA AAA ACC CAC ACT CAT ATC AAT ATA (Imamura et al., 2006). The cycling conditions were: 94^0C for 10 min, followed by 40 cycles of 94^0C for 60s, 50^0C for 60s and 72^0C for 40s with a final 5 min extension at 72^0C. PCR products (367 bp amplicon) were gel-purified and cloned into the pCRII-TOPO TA vector (invitrogen). Approximately 30 clones were picked and sequenced with M13 forward and reverse primers. Sequences characterised by incomplete bisulphite conversion were discarded from the analysis.

2.8 Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE)

Regions of active chromatin are characterized by their hypersensitivity to nucleases (Wu et al., 1979), which in turn correlate with nucleosomes-depleted regions (Boeger et al., 2003). Notably, the clearance of nucleosomes from active regulatory regions is a conserved mechanism in eukaryotes (Wallrath et al., 1994). Recently it has been shown that active promoters/transcription start sites are preferentially isolated using FAIRE (Giresi et al., 2007; Nagy et al., 2003).

2.8.1 Preparation of embryos and cells

FAIRE was performed as described (Giresi et al., 2007) with minor modifications. Embryos from MF1 inter-crosses were dissected and staged according to Downs and Davies classification as described above (Section 2.3.1). Each biological replicate consisted of 27-30 pooled E7.5 embryos or 20-22 E8.5 embryos. Extra-embryonic tissue (including the allantois containing primordial germ cells) was removed from embryos, and the embryo proper was dissociated using 0.5% trypsin/2.5% pancreatin/PBS. For MEFs or ES cells, $1x10^7$ cells were used per replicate. For analysis of E8.5 explants, batches of 20-22 embryos were dissociated using 0.5% trypsin/2.5% pancreatin PBS and cells cultured in EpiSC conditions in the presence or absence of 1mg/ml Doxycycline. The next day, explants were dissociated with trypsin, replated on non-gelatinised tissue culture dishes for 15 min to deplete the feeders and collected for FAIRE analysis.

2.8.2 Chromatin preparation

ES cells, MEFs or embryonic tissue was resuspended in 3ml of pre-warmed culture media (GMEMβ/FCS/LIF, GMEMβ/FCS and DMEM-F12β/KOSR/Activin/bFGF respectively) and cross-linked for 10' at RT with 1% formaldehyde (Sigma). The cross-linking reaction was stopped by adding 0.125mM glycine for 5 min at RT. Cells were pelleted (3 min, 1300rpm, 4°C) and washed twice with ice-cold PBS 1X (Invitrogen). Cell pellets were then vigorously resuspended in 300µl of swelling buffer (5mM Pipes pH8, 85mM KCl) freshly supplemented with 1X protease inhibitor cocktail (Roche) and 0.5% NP-40. After 20 minutes on ice with occasional shaking, nuclei were centrifuged (1500rpm in 15ml conical tubes (Falcon-BD), 10 min, 4°C) and resuspended in 0.5ml (ES cells & MEFs) or 0.1ml (embryonic tissue) of TSE150 (0.1% SDS, 1% Triton, 2mM EDTA, 20mM Tris-HCl pH8, 150mM NaCl) buffer, freshly supplemented with 1X protease inhibitor cocktail. Samples were then sonicated at 4°C (15ml conical tubes for cultured cells and 1.8ml microcentrifuge tubes for embryo-derived nuclei) using a Bioruptor (Diagenode) for 3-4 cycles of 10 min divided into 30 seconds ON-30 seconds OFF sub-cycles at maximum power. Chromatin was then microcentrifuged for 30min at 4°C (ALC PK120, Annita). Soluble chromatin was used immediately or stored at -80°C. Twenty microlitres were set apart and used to quantify the chromatin concentration and the size of the DNA fragments on a 1.5% agarose gel.

2.8.3 Isolation of protein-free DNA fragments

For isolation of nucleosome-depleted INPUT DNA, crosslinking was reversed by adding 250µl of TE/1%w/v SDS and incubated at 65° overnight. The

next day the following solutions were added, 5µl of Proteinase K (20mg/ml), 5µl glycogen (2µg/ml) and 240µl TE. The INPUT DNA was then placed at 37°C for 2hrs. The INPUT DNA was extracted with phenol-chloroform followed by ethanol precipitation and RNase A (100 µg/ml) treatment. Cross-linked samples were incubated at 65°C for 2hrs to ensure that no DNA–DNA crosslinks were maintained. Samples were amplified using a LightCycler 480 (Roche) and LightCycler 480 SYBR Green 1 Master (Roche).

Table 2. 4 Primers used for FAIRE

Target region	Sequence
Nanog (sense)	TGG CCT TCA GAT AGG CTG AT
Nanog (antisense)	CAA GAA GTC AGA AGG AAG TGA GC
Sox2 (Sense)	AGG GCT GGG AGA AAG AAG AG
Sox2 (antisense)	CCG CGA TTG TTG TGA TTA GTT
Oct4 (Distal Enhancer; sense)	AGA GTG CTG TCT AGG CCT TA
Oct4 (Distal Enhancer; antisense)	CCA GAA CTC TCA ACC TCC CT
Oct4 (Proximal Enhancer; sense)	GGG AAG CAG GGT ATC TCC AT
Oct4 (Proximal Enhancer; antisense)	TCC CCT CAC ACA AGA CTT CC
Tsix (sense)	GCG CTT GCA GGT ACT TTT
Tsix (antisense)	AAG AGC CTT AGG TCC CGC C
Actin (sense)	CCG TTC CGA AAG TTG CCT T
Actin (antisense)	CGC CGC CGG GTT TTA TA

Chapter 3

Role of Sox2 and FGF signaling in pluripotent cells

3.1 Introduction

Several studies have shown that under self-renewal conditions, mouse ES cells exhibit considerable heterogeneity in the levels of factors such as Nanog, Stella, Rex and Pecam1 (Chambers et al., 2007; Furusawa et al., 2006; Hayashi et al., 2008; Toyooka et al., 2008). Nanog fluctuations in ES cells occur in the undifferentiated fraction of the population (Chambers et al., 2007). ES cells with low levels of Nanog show a pre-disposition towards differentiation, whereas high levels of Nanog present a bias towards self-renewal (Chambers et al., 2003; Chambers et al., 2007). Notably, different cellular substates within the undifferentiated compartment are reversible, thus, a cell that has low Nanog expression can give rise to a high Nanog expressing cell and *vice versa* (Chambers et al., 2007).

In pre-implantation embryos FGF signaling is necessary for the specification of the lineages that make up the ICM (Arman et al., 1998; Cheng et al., 1998). FGF-Erk signaling pathway has been reported to have an effect on the ability of cells to differentiate (Burdon et al., 1999; Kunath et al., 2007; Stavridis and Smith, 2003).

FGF4 transcription is directly activated *in vitro* by the synergistic interaction between Oct4 and Sox2 on the FGF4 enhancer (Dailey et al., 1994; Yuan et al., 1995). Oct4 expression in the ICM is not regionalized during the specification of the epiblast/hypoblast, whereas Sox2 and Nanog expression is restricted to the nascent epiblast (Guo et al., 2010). Therefore, it is conceivable that paracrine signaling, involving FGF4 secretion by epiblast cells, is crucial for the commitment of cells that express FGFr2 into primitive endoderm. In support of this hypothesis, when 8-cell embryos are cultured in the presence of inhibitors of the FGF/Erk pathway, the ICM of these embryos is composed exclusively of $Nanog^{+}$ cells (Nichols et al., 2009). In summary, that the specification of the epiblast/hypoblast may involve a differential expression of *FGF4* and *FGFr2* in progenitor cells (Chazaud et al., 2006; Nichols and Smith, 2009; Yamanaka et al., 2010). It has also been suggested that paracrine FGF signaling in ES cells may play a role in priming cells for differentiation. Nanog plays a fundamental role in regulating ES cell self-renewal, therefore, it is likely that FGF signaling influences fate decisions through modulations of Nanog levels. However, the precise relationship between Nanog and FGF signaling remain poorly understood.

Recent studies suggest that Nanog heterogeneity is influenced by the levels of Oct4 (Violetta Karwacki-Neisius, PhD thesis UoE). The level of Oct4 is a critical factor for the specification of fate in mouse ES cells. Normal diploid expression of Oct4 in ES cells is necessary to sustain self-renewal, a two fold increase in the levels of this transcription factor causes differentiation of a proportion of the population into primitive endoderm, while repression induces dedifferentiation into trophectoderm. Oct4 heterozygote ES cells, which have lower levels of Oct4, are

considerably less heterogeneous in Nanog expression than wildtype ES cells, with the vast majority of the undifferentiated population expressing high levels of Nanog (Violetta Karwacki-Neisius, PhD thesis UoE). Oct4$^{+/-}$ ES cells display a bias towards self-renewal, consistent with the high, homogenous Nanog expression. Importantly, the reduced Nanog heterogeneity observed in Oct4$^{+/-}$ can be manipulated to mimic the heterogeneity observed in wildtype ES cells by artificially increasing the Oct4 levels (Violetta Karwacki-Neisius, PhD thesis UoE).

The majority of the pluripotency-associated genes contain enhancers that possess Oct4/Sox2 binding motifs (Chen et al., 2008). Oct4 and Sox2 bind independently to their motifs, however, their binding to DNA is stabilized by protein-protein interaction in the ternary complex (Ambrosetti et al., 1997; Ambrosetti et al., 2000; Remenyi et al., 2003). Due to the close functional relationship between Oct4 and Sox2 it is feasible that the pluripotency network is susceptible to variation in Sox2 levels.

3.2 Sox2 is essential for ES cell self-renewal

Sox2 deletion in the embryo is lethal at the post-implantation stage. Detail analysis of *Sox2*$^{-/-}$ mutants reveals a role of Sox2 in the maintenance of the epiblast and extra-embryonic ectoderm, as the only surviving cells at this stage are giant trophoblast cells and extra-embryonic endoderm (Avilion et al., 2003). Sox2 knockdown by siRNA in ES cells suggest that Sox2 is required to prevent ES cell differentiation into both the trophectoderm and epiblast-derived lineages (Ivanova et al., 2006). A more rigorous analysis showed that inducible genetic deletion of

Sox2 in ES cells results in differentiation of ES cells into trophectodermal cells, similar to Oct4 deletion (Masui et al., 2007). Interestingly, the phenotype observed following the genetic deletion of Sox2 could be rescued not only with ectopic Sox2, but also by an Oct4 transgene (Masui et al., 2007). Together these results suggest that Sox2 functions in ES cells to stabilize Oct4 expression. This has been hypothesized to occur by modulation of positive (Nr5a2) and negative (Nr2f2) regulators of the *Oct4* (Masui et al., 2007).

To clarify the role of Sox2 in ES cell self-renewal, ES cell lines carrying mutations in the Sox2 gene were used ($Sox2^{+/Bgeo}$ and $Sox2^{fl/Bgeo}$;Cre-ER^{T2} ES cells were a kind gift from Silvia Nicolis, University of Milano-Bicocca). Gene targeting was used to replace the Sox2 ORF with a fusion of β-galactosidase and the neomycin-resistance gene (Figure 3.1A) (Zappone et al., 2000). The $Sox2^{+/Bgeo}$ ES cell line was further modified by the electroporation of a targeting vector containing a floxed-Sox2 sequence (Figure 3.1A) (Favaro et al., 2009). Finally, to achieve efficient conditional deletion of Sox2, a random integration of a CAG-driven Cre-ER^{T2} was performed (Figure 3.1A).

Activation of the tamoxifen inducible Cre-recombinase in $Sox2^{fl/Bgeo}$;Cre-ER^{T2} (Sox2CKO) ES cells for 12h led to the complete loss of Sox2 protein (Figure 3.1B). Loss of Sox2 protein resulted in a marked reduction in the protein levels observed for Oct4, Nanog and Esrrb (Figure 3.1B). Complete disappearance of Oct4, Nanog

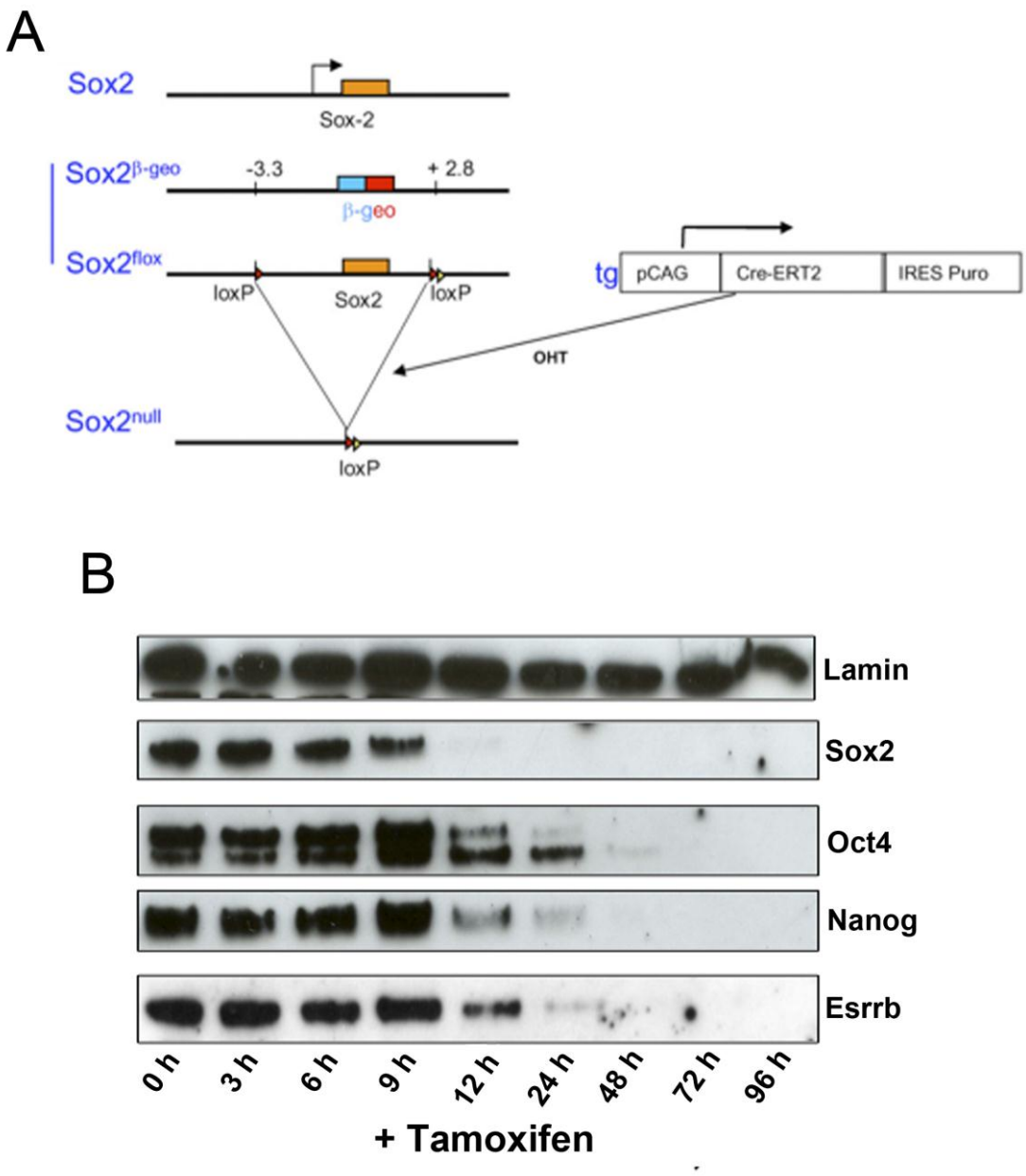


Figure 3. 1 Sox2 mutant ES cells

(A) Strategy used for the construction of $Sox2^{Bgeo/+}$ and Sox2CKO. Sox2 mutants were a kind gift from Silvia Nicolis.

(B) Timecourse of Sox2 deletion in Sox2CKO ES cells. Western blot depicting the changes in protein levels of Sox2, Oct4, Nanog and Esrrb.

and Esrrb is observed 48h after the induction of Cre recombinase. Moreover, significant reduction of the Sox2 transcript can be detected as early as 3h after tamoxifen treatment (Figure 3.2).

Oct4 and Nanog transcripts are reduced by 48h and become undetectable by 72h after tamoxifen treatment (Figure 3.2). In the case of Esrrb, the transcript is reduced by 24h and becomes undetectable by 48h after tamoxifen treatment (Figure 3.2). Klf4 transcript increases after 24h of the start of tamoxifen treatment until it is subsequently downregulated after 72h (Figure 3.3). Klf2 and Nr5a2 transcripts show a similar response to Sox2 deletion, displaying a progressive decrease in the level of the transcripts (Figure 3.3). The level of Tbx3 transcript is halved after 72h of tamoxifen treatment, decreasing even further by 96h (Figure 3.3).

To assess the role of Sox2 in ES cell self-renewal, Sox2CKO ES cells were seeded at clonal density in the presence or absence of tamoxifen. Sox2CKO ES cells cultured in FCS/LIF containing media, in the absence of tamoxifen treatment, yielded around 300 AP+ colonies after 7 days of culture (Figure 3.4). In contrast, a complete loss of AP+ colonies was observed after tamoxifen treatment for the duration of the assay (Figure 3.4). A shorter exposure to tamoxifen (30h) yielded a small number of AP+ colonies (~10). The deletion of Sox2 in a clonal assay was subsequently performed in N2B27/2i/LIF. Under such conditions, Sox2CKO ES cells seeded at clonal density, yielded around 600 AP+ colonies (Figure 3.4). Tamoxifen treatment for 30h significantly decreased the ability of Sox2CKO ES cells to form AP+ colonies in N2B27 supplemented with 2i/LIF (Figure 3.4). Next, it was

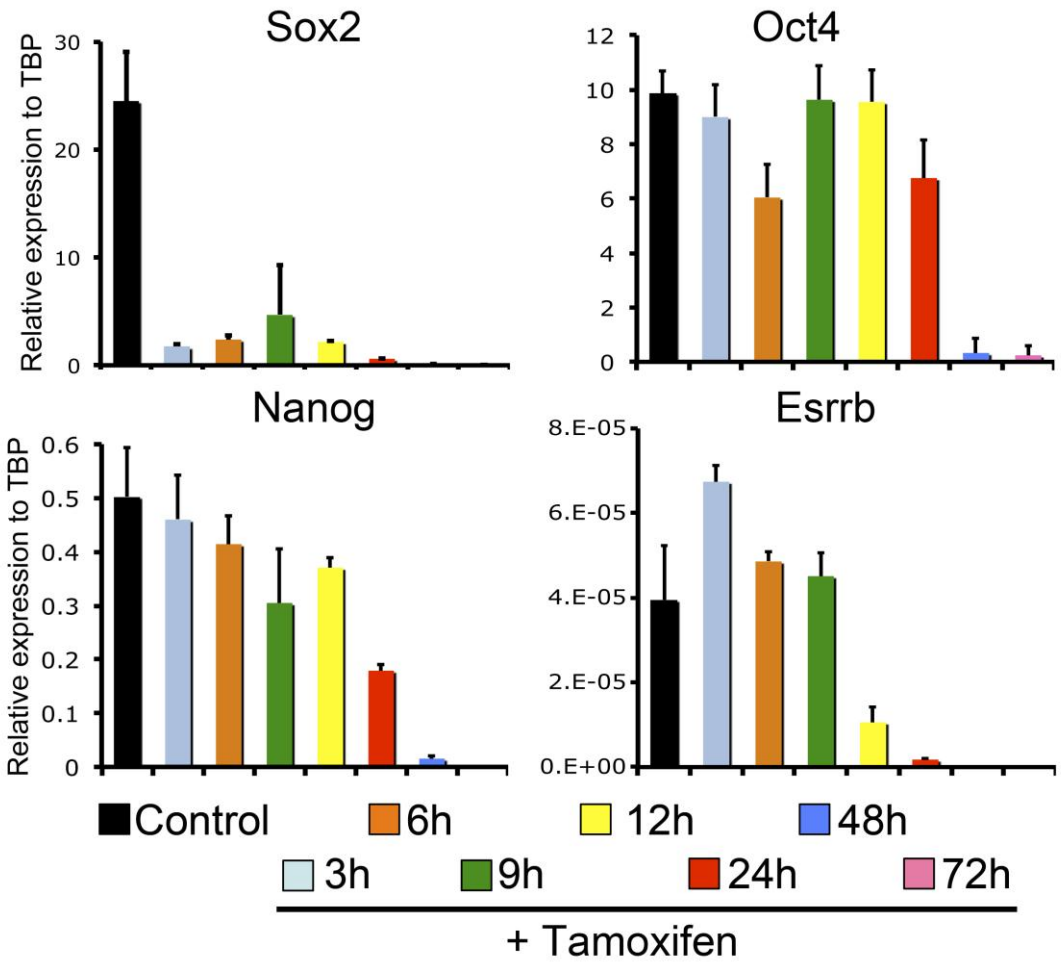


Figure 3. 2 Expression of pluripotency factors following Sox2 deletion.

Gene expression analysis of Sox2CKO following tamoxifen treatment with the indicated times. qPCR analyses were determined relative to TATA box-binding protein (TBP). All qPCR results are shown as the average of distinct experiments performed in at least 2 independent RNA preparations.

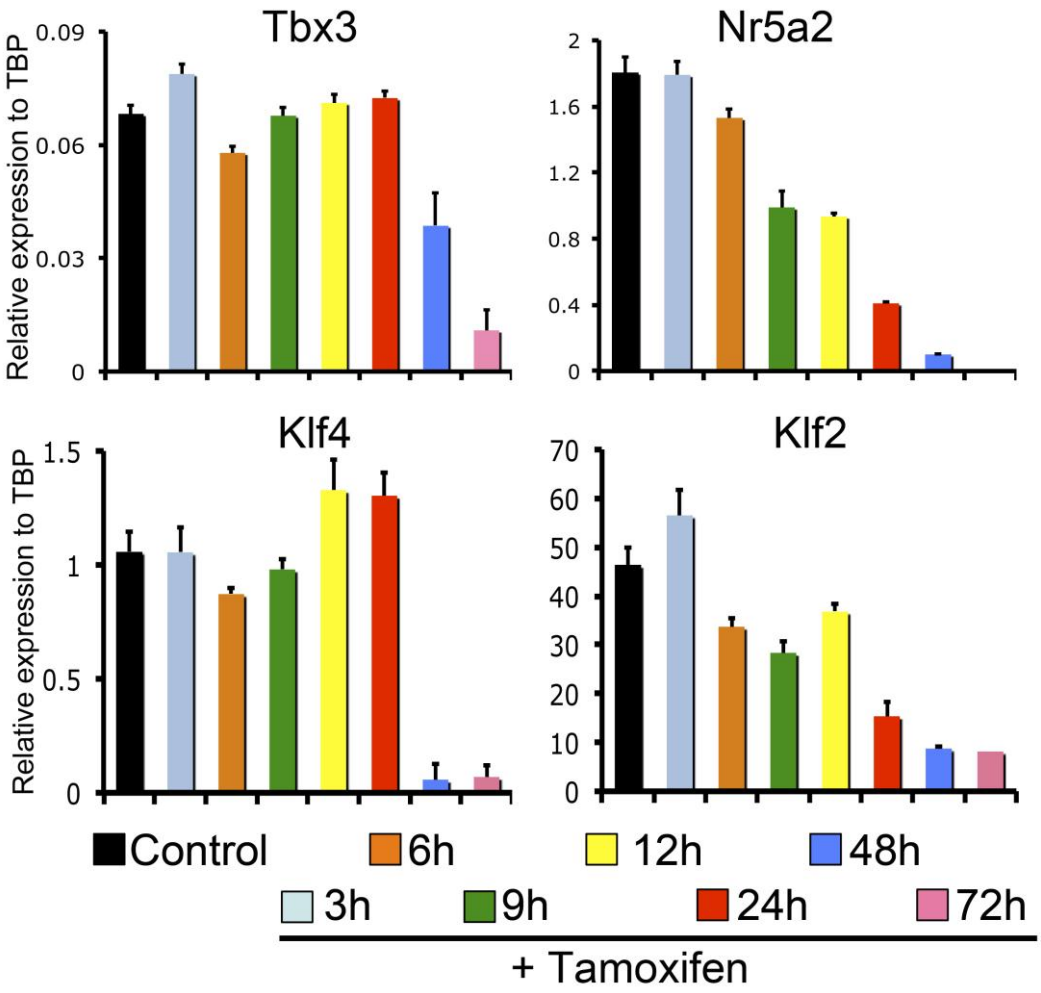


Figure 3. 3 Sox2 deletion leads to the downregulation of pluripotency associated factors.

Gene expression analysis of Sox2CKO following tamoxifen treatment with the indicated times. qPCR analyses were determined relative to TATA box-binding protein (TBP). All qPCR results are shown as the average of distinct experiments performed in at least 2 independent RNA preparations.

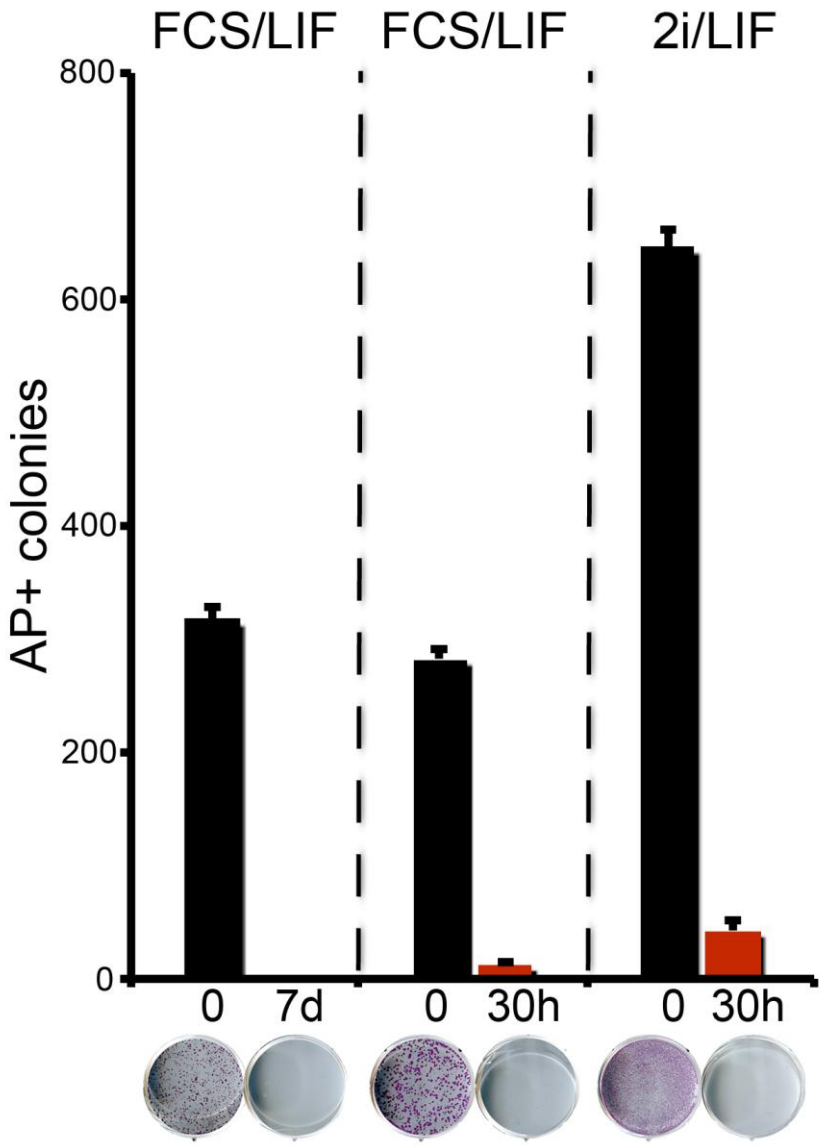


Figure 3. 4 Effect of Sox2 deletion in ES cell self-renewal.

Clonal assay of Sox2CKO ES cells. Sox2CKO were seeded at clonal density under different exposure periods to tamoxifen (top and middle panels) and in different media (middle and lower panels).

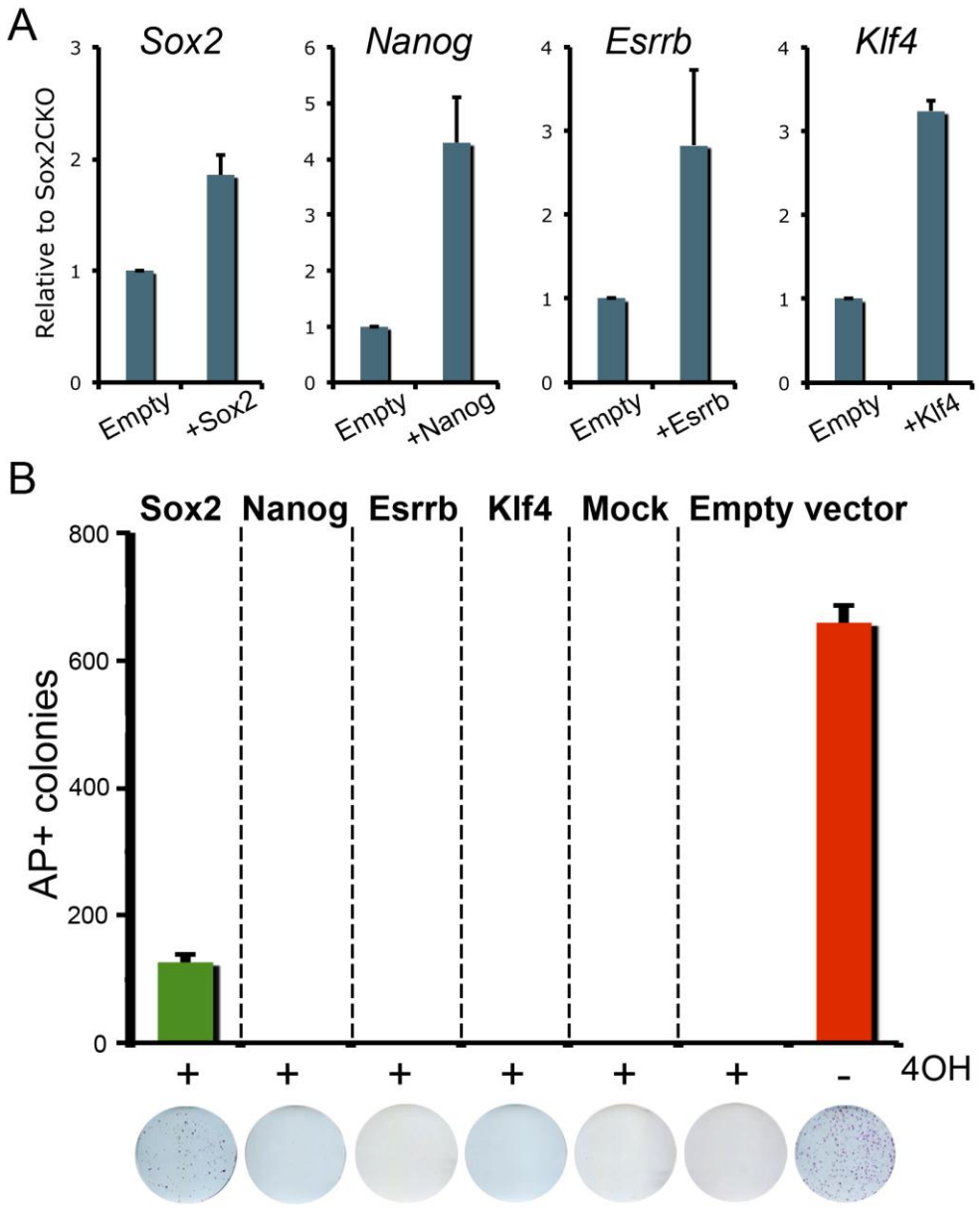


Figure 3. 5 Rescue of Sox2CKO ES cells with exogenous Sox2.

(A) Expression of Sox2, Nanog, Esrrb and Klf4 in Sox2CKO ES cells. Plasmids containing either empty vector, Sox2, Nanog, Esrrb or Klf4 were electroporated in Sox2CKO. Following electroporation the cells were seeded at a density of 1 x 10^6 into a 100mm dish and selection was started. After 10 days, total mRNA was isolated from the populations. Error bars are the SD of 3 technical replicates.

(B) Plasmids containing either empty vector, Sox2, Nanog, Esrrb or Klf4 were electroporated in Sox2CKO. Following electroporation the cells were seeded at a density of 1 x 10^6 into a 100mm dish. The following day, selection and tamoxifen treatments were initiated. Plates were stained for alkaline phosphatase after 7 days of culture.

tested whether Sox2 deficiency in ES cells could be rescued with other members of the pluripotency network (Figure 3.5). Although populations of ES cells overexpressing Sox2, Nanog, Esrrb or Klf4 could be generated (Figure 3.5A), only Sox2 was able to rescue the phenotype observed following Sox2 deletion in ES cells (Figure 3.5B).

3.3 Sox2 is essential for EpiSC self-renewal

Sox2CKO ES cells were differentiated *in vitro* into EpiSC by a medium switch from GMEMβ/FCS/LIF to N2B27/Activin/FGF. Sox2CKO EpiSC retained expression of the core pluripotency factors Oct4, Sox2 and Nanog, while upregulating the early marker for differentiation FGF5. Moreover, Sox2CKO downregulated the ES cell-specific factors Klf4, Klf2 and Esrrb (Figure 3.6). Sox2CKO cells also displayed bona fide EpiSC morphology in the absence of tamoxifen (Figure 3.7A). Interestingly, tamoxifen treatment of Sox2CKO EpiSC promoted differentiation and loss of self-renewal ability (Figure 3.7A). Protein analysis indicated that Sox2 protein is lost 48h after Cre-induction in Sox2CKO EpiSC (Figure 3.7B).

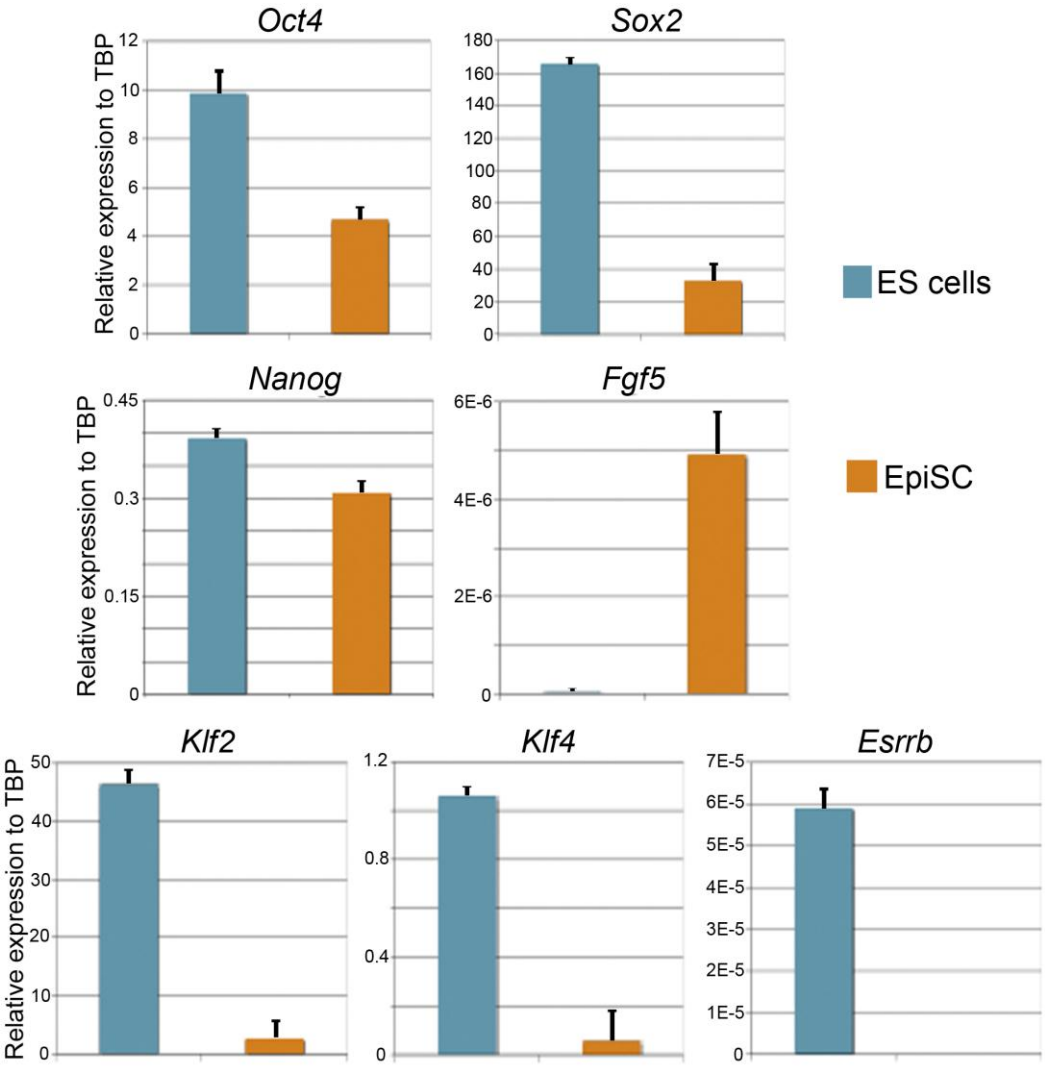


Figure 3. 6Sox2CKO cells can make EpiSC.

Marker expression analysis in Sox2CKO ES cells and EpiSC. qPCR analyses were determined relative to TATA box-binding protein (TBP). qPCR results are shown as the average of distinct experiments performed in at least 2 independent RNA preparations.

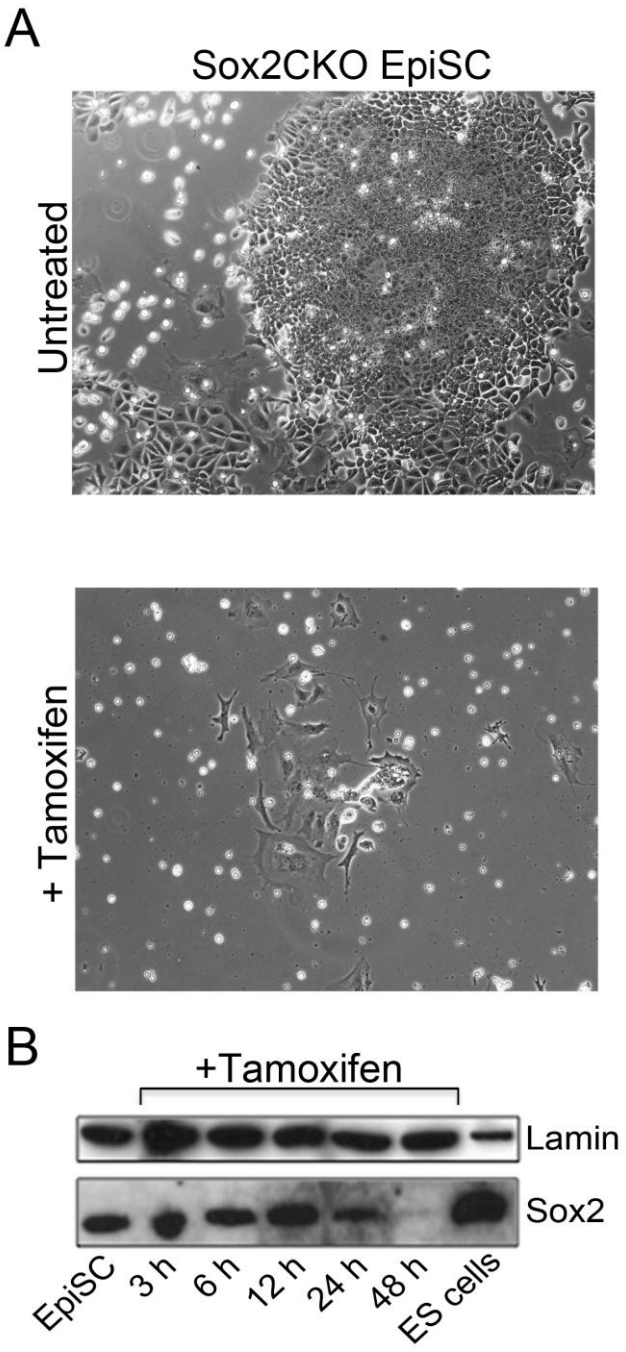


Figure 3. 7 Sox2 deletion in Sox2KO EpiSC.

(A) Brightfield images depicting the morphological changes occurring 48hrs after Sox2 deletion in EpiSC.

(B) Western blot displaying the Sox2 protein level changes over 48h, following the tamoxifen-induced deletion of Sox2 in EpiSC.

3.4 Role of FGF and Sox2 on Nanog heterogeneity

To study the effect of reduced Sox2 and Fgf signaling on Nanog expression, several ES cell lines were generated (Figure 3.8A). $Sox2^{+/Bgeo}$ (cells were a kind gift from Silvia Nicolis, University of Milano-Bicocca) and $FGF4^{puro/Bgeo}$ (Wilder et al., 1997) ES cells were targeted with an eGFP at the *Nanog* AUG codon (Chambers et al., 2007). For each mutant ES cell lines 4 clones were analysed and representative results are displayed throughout the chapter 3. Staining for Oct4 protein in TNG and SONG ES cells showed that the undifferentiated fraction of the population displays a heterogeneous expression of *Nanog*:GFP (Figure 3.8B). Interestingly, the undifferentiated fraction (Oct4+ cells) of FNG ES cells displayed reduced heterogeneity in *Nanog*:GFP expression (Figure 3.8B).

Quantitive PCR was used to compare the level of expression of several pluripotency associated transcription factors in TNG, SONG and FNG ES cells (Figure 3.9). Sox2 transcript was significantly lower in Sox2 heterozygous ES cells when compared to ES cells with intact Sox2 alleles (Figure 3.9). Consistent with these observations, the expression of the Sox2 target gene, Nr5a2, was significantly reduced in SONG (Figure 3.9). Interestingly, *FGF4*$^{-/-}$ ES cells a 2-fold increase in the level of Klf2 and Klf4 transcripts (Figure 3.9). FNG ES cells also displayed a modest increase in Nanog transcription (Figure 3.9). The Oct4 transcript levels in SONG and FNG ES cells are slightly lower when compared to TNG ES cells (Figure 3.9).

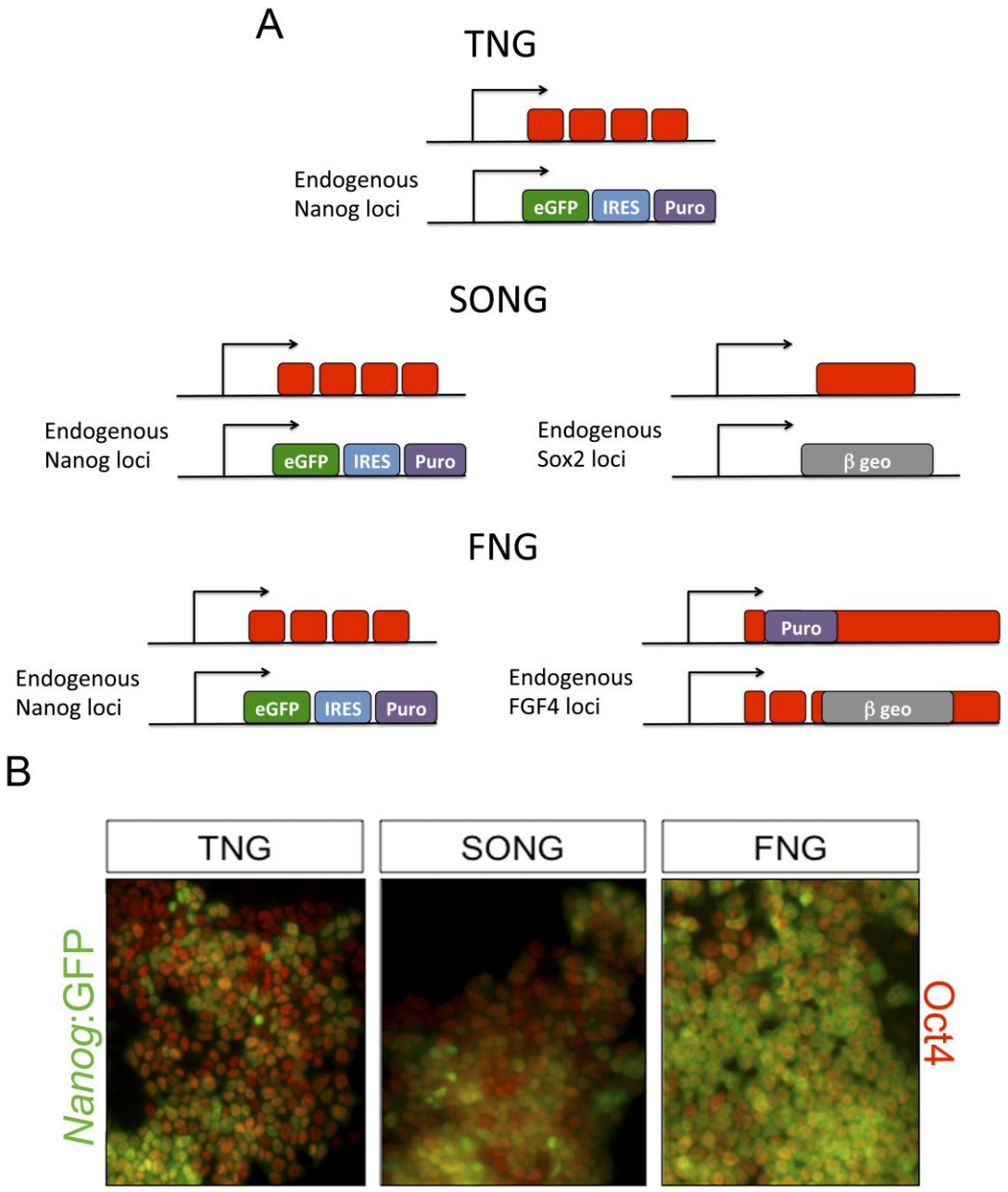


Figure 3. 8 Mutant ES lines to study Nanog heterogeneity.

(A) Schemes showing the genetic modifications in TNG ES cells ($Nanog^{+/GFP}$), SONG ES cells ($Nanog^{+/GFP}$; $Sox2^{+/Bgeo}$) and FNG ES cells ($Nanog^{+/GFP}$; $FGF4^{Puro/Bgeo}$).

(B) *Nanog*:GFP expression and Oct4 staining in TNG, SONG and FNG ES cell lines.

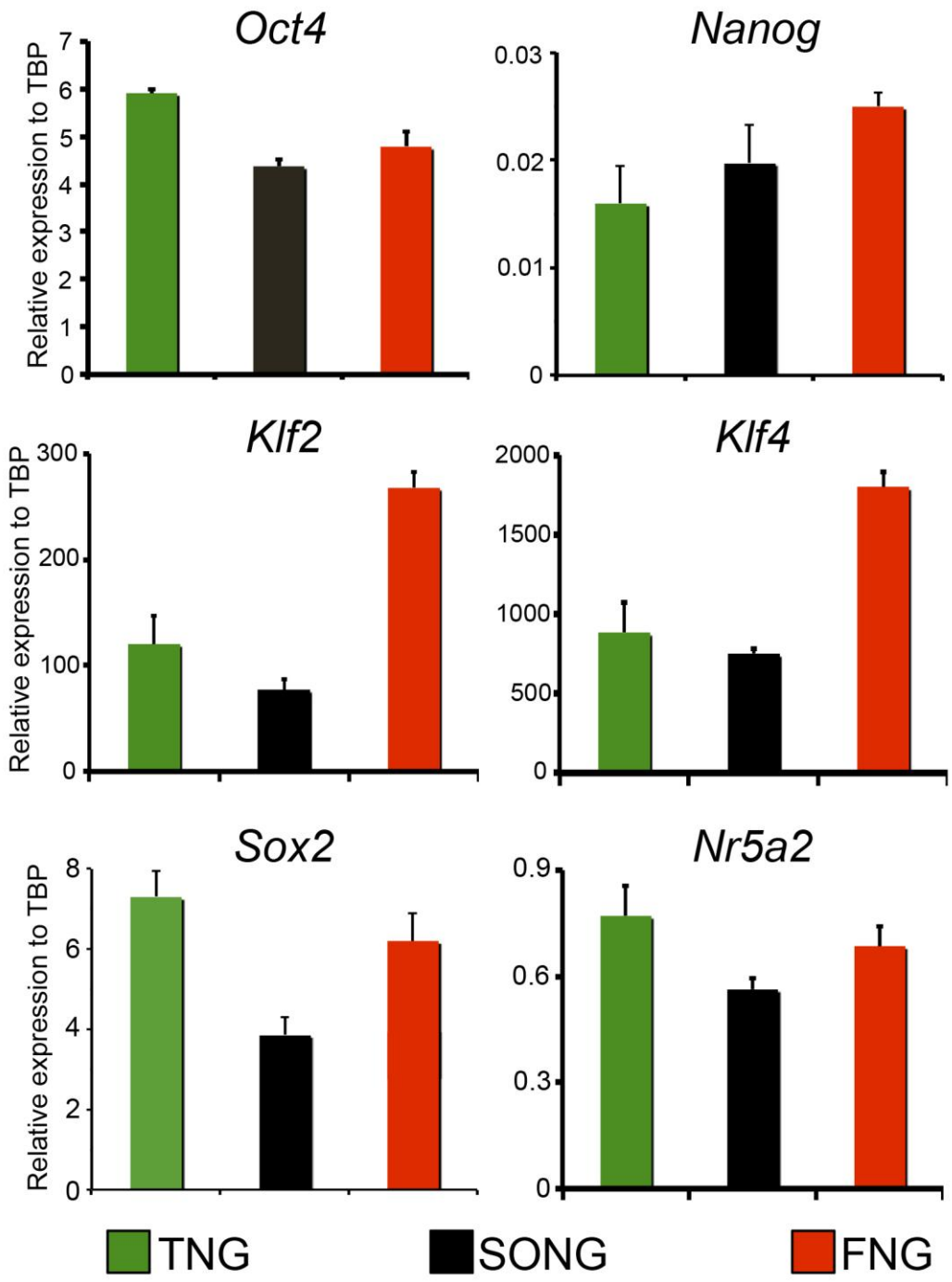


Figure 3. 9 Pluripotency gene expression in TNG, SONG and FNG ES cells.

Pluripotency marker expression in TNG, SONG and FNG in the absence of selection for Nanog expression (Puromycin). qPCR analyses were determined relative to TATA box-binding protein (TBP). All qPCR results are shown as the average of distinct experiments performed in at least 2 independent RNA preparations.

TNG, SONG and FNG ES cells express Puromycin resistance from the *Nanog* locus, therefore drug selection allows for propagation of pure populations of $Nanog^{+}$ ES cells (Figure 3.10). Pre-selected TNG, SONG and FNG ES cells were seeded at a low density in 6-well plates, without selection, in media containing FCS/LIF and in the presence or absence of recombinant basic FGF (bFGF). On the specified days the emerging populations were quantified for expression of SSEA-1 and *Nanog*:GFP using flow cytometry (Figure 3.10). Following two days of culture under these conditions, a small number of GFP^{low} cells began to emerge from the undifferentiated ($SSEA\text{-}1^{+}$) fraction of TNG ES cells (Figure 3.10, top panels). A more pronounced shift in the proportion of GFP^{high}/GFP^{low} in the undifferentiated fraction of the population was observed at day 4 (Figure 3.10, top panels). By day 7, a considerable proportion of the $SSEA\text{-}1^{+}$ subpopulation had lost expression of *Nanog*:GFP (Figure 3.10, top panels). Supplementation of exogenous bFGF resulted in a modest increase in the number of GFP^{low} cells within the undifferentiated subpopulation (Figure 3.10, top panels). SONG ES cells displayed a broader *Nanog*:GFP distribution than TNG ES cells throughout the assay (Figure 3.10, middle panels). Moreover, SONG ES cells showed a faster appearance of GFP^{low} cells than TNG ES cells (Figure 3.10, middle panels). Similar to the observation made using TNG ES cells, supplementation of SONG ES cells with exogenous bFGF, had a modest effect on *Nanog*:GFP heterogeneity (Figure 3.10, middle panels). In stark contrast, FNG ES cells do not display an increase in the proportion of GFP^{low} cells (Figure 3.10, lower panels). *Nanog*:GFP expression in FNG ES cells remains homogenous for the duration of the assay (Figure 3.10, lower panels). Restoring FGF signaling in FNG ES cells by the supplementation of bFGF, promoted the

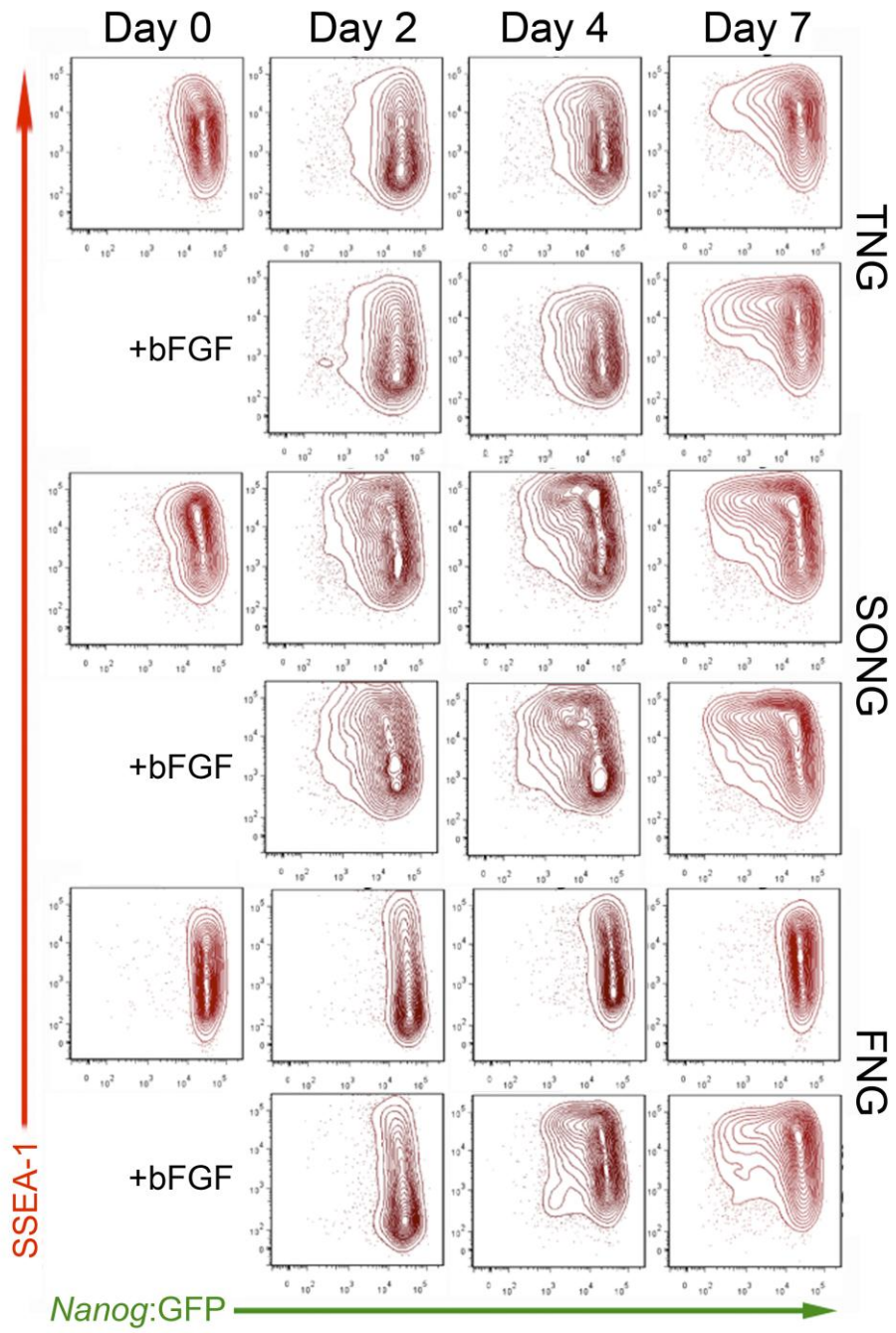


Figure 3. 10 *Nanog*:GFP expression in TNG, SONG and FNG ES cells.

TNG, SONG and FNG ES cells, pre-selected for homogenous Nanog expression with Puromycin, were seeded in 6-well plates at low density. The following day, defined as day 0, selection was removed and the media was replaced with GMEMβ/FCS/LIF in the presence or absence of bFGF. Cells were then stained for SSEA-1 and analysed by flow cytometry on days 0, 2, 4 and 7. Representative FACS plots are shown. At least 4 biological replicates were performed for each FACS experiment.

appearance of SSEA-1^{+}/GFP^{low} cells between days 2-4 of treatment (Figure 3.10, lower panels).

In flow cytometry, two measures are generally taken from a fluorescent distribution, intensity and spread. The intensity of a distribution can be represented arithmetically by the mean or median. If the data has been displayed on a logarithmic scale, the geometric mean is preferred ((Shapiro, 2003) Figure 3.1A). The spread of a distribution is usually displayed as the Standard Deviation (SD). However, the coefficient of variation (CV) is a useful alternative as takes into account the biological variation and the measuring variation (CV = SD/mean). The CV is a function that allows the quantitative comparison of the heterogeneity observed in a fluorescent distribution.

TNG, SONG and FNG ES cells were seeded at low density in GMEMβ/FCS/LIF alone or supplemented with either a GSK3 (CHIR99021) or MEK (PD0325901) inhibitor. Each day the levels of *Nanog*:GFP were measured using flow cytometry. The CV and median were calculated for each sample and the average of four biological repeats were plotted (Figure 3.11). Upon release from selection, *Nanog*:GFP^{low} cells were readily generated in cultures of TNG ES cells at low density in GMEMβ/FCS/LIF (Figure 3.10) (Chambers et al., 2007). As expected, the CV of *Nanog*:GFP in TNG ES cells showed a consistent increase for the duration of the experiment (Figure 3.11A, left panel). Culture of TNG ES cells in GMEMβ/FCS/LIF supplemented with a GSK3 inhibitor reduced significantly the increase in the CV of *Nanog*:GFP, when compared to the untreated samples (Figure 3.11A, left panel).

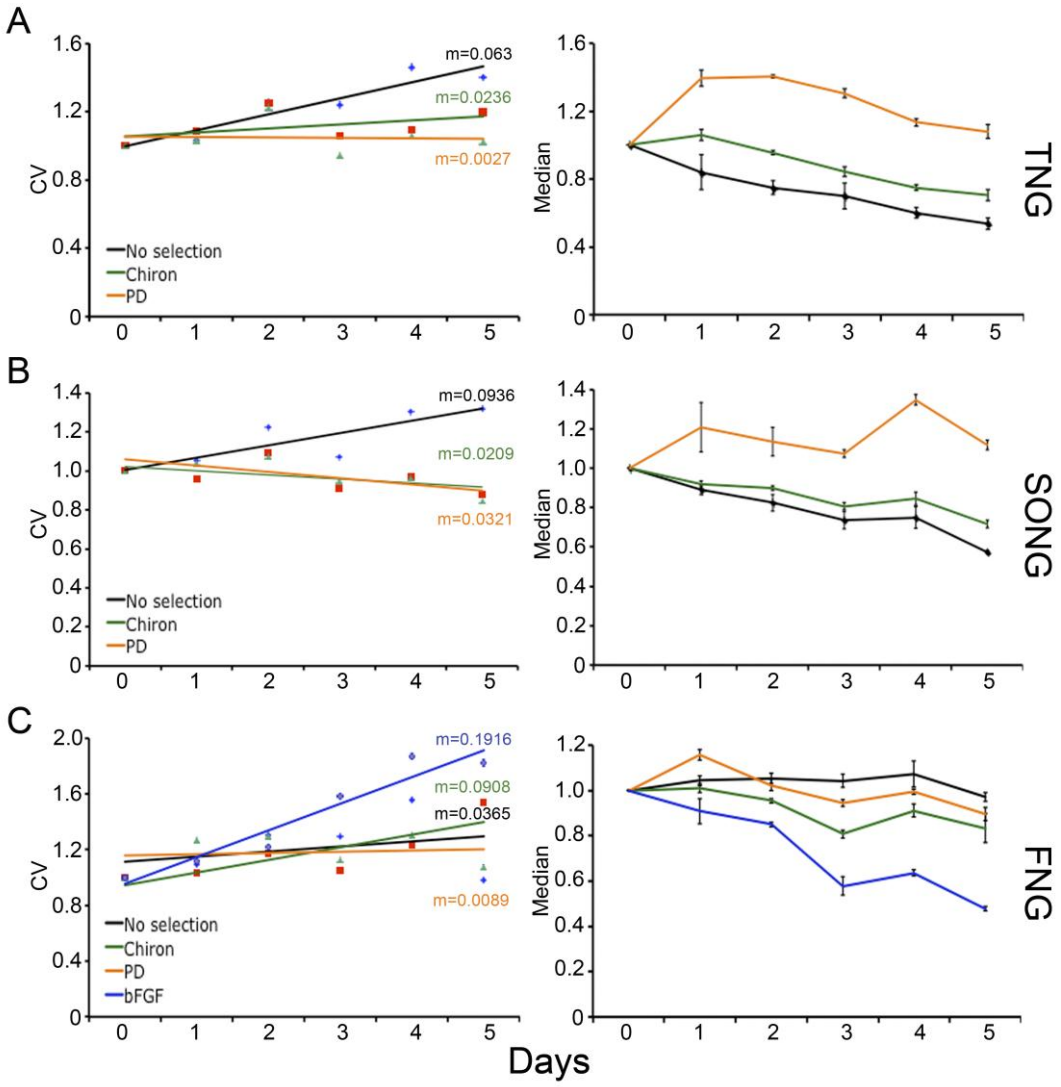


Figure 3. 11 Changes in Nanog heterogeneity following MEK and GSK3 modulation in TNG, SONG and FNG ES cells.

(A) CV and median analysis of *Nanog*:GFP in TNG cells following MEK (PD0325901) and GSK3 (CHIR99021) modulation.

(B) CV and median analysis of *Nanog*:GFP in SONG cells following MEK (PD0325901) and GSK3 (CHIR99021) modulation.

(C) CV and median analysis of *Nanog*:GFP in FNG cells following MEK (PD0325901) and GSK3 (CHIR99021) inhibition.

Strikingly, the CV of *Nanog*:GFP from TNG ES cells cultured in GMEMβ/FCS/LIF supplemented with a MEK inhibitor (PD0325901) showed a consistent decrease (Figure 3.11A, left panel). Furthermore, culture of TNG ES cells at low density in GMEMβ/FCS/LIF alone or supplemented with a GSK3 inhibitor (CHIR99021) resulted in a progressive decrease in the median of *Nanog*:GFP (Figure 3.11A, right panel). Exposure of TNG ES cells to the MEK inhibitor (PD0325901) in the presence of serum containing medium resulted in an increase in *Nanog*:GFP (Figure 3.11, right panel).

Similar to TNG cells, SONG ES cells readily generated *Nanog*:GFP^{low} cells when seeded at low density in GMEMβ/FCS/LIF (Figure 3.10). SONG ES cells also displayed a growth in the CV of *Nanog*:GFP under similar conditions (Figure 3.11B, left panel). Inhibition of either GSK3 (CHIR99021) or MEK (PD0325901) prevented an increase in heterogeneity of *Nanog*:GFP in SONG ES cells (Figure 3.11B, left panel). The overall levels of *Nanog*:GFP showed a gradual decrease with time when SONG ES cells were plated at low density in GMEMβ/FCS/LIF alone or supplemented with a GSK3 inhibitor (Figure 3.11B, right panel). In contrast, SONG ES cells grown in the presence of a MEK inhibitor (PD0325901) showed an overall increase in the median of *Nanog*:GFP (Figure 3.11B, right panel).

ES cells lacking a functional copy of the FGF4 gene failed to generate the *Nanog*:GFP^{low} cells at the same rate as controls ES cell lines (Figure 3.10). Restoring FGF signaling, by the addition of exogenous bFGF, rescues the ability of FNG ES

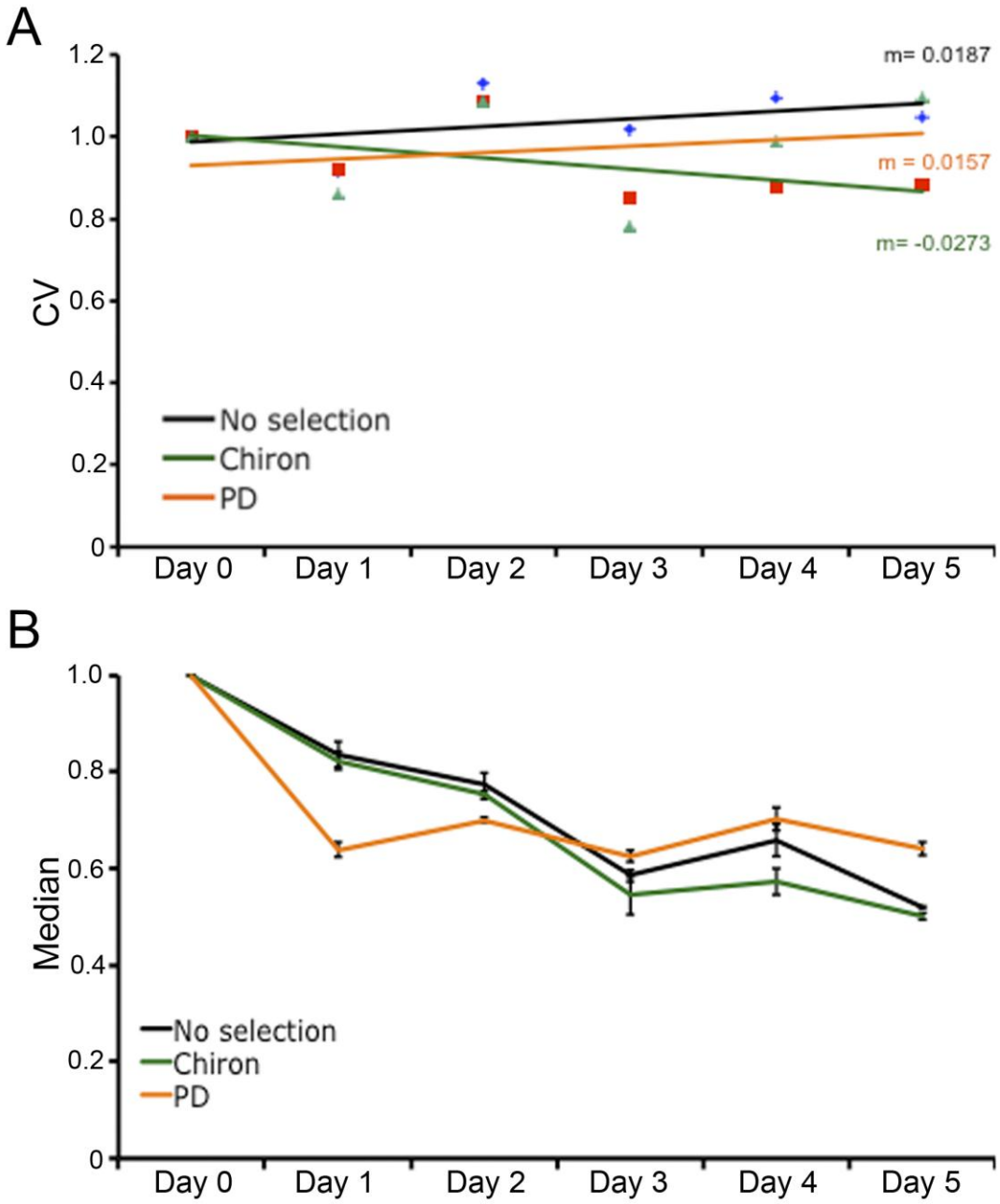


Figure 3. 12 Effect of GSK3 and MEK inhibition on the CV and median of *Oct4*:GFP.

(A) Changes in CV of *Oct4*:GFP in Oct4GiP ES cells after removal of selection.

(B) Observed changes in the median of *Oct4*:GFP in Oct4GiP ES cells after removal of selection.

cells to generate undifferentiated *Nanog*:GFP^{low} cells (Figure 3.10; Figure 3.11C, left panel). Culture of FNG ES cells at low density in GMEMβ/FCS/LIF alone or supplemented with a PD0325901 failed to display a significant change in the CV of *Nanog*:GFP (Figure 3.11C, left panel). On the other hand, FNG ES cells grown in GMEMβ/FCS/LIF supplemented with either a GSK3 inhibitor or bFGF showed a consistent increase in the CV of *Nanog*:GFP (Figure 3.11C, left panel). Propagation of FNG ES cells at low density in GMEMβ/FCS/LIF supplemented with bFGF promoted a progressive decrease in the median of *Nanog*:GFP (Figure 3.11C, right panel). FNG ES cells cultured in GMEMβ/FCS/LIF alone or in the presence of either a GSK3 (CHIR99021) or MEK (PD0325901) inhibitor did not show a significant change in the median of *Nanog*:GFP (Figure 3.11C, right panel).

Oct4 levels are critical in the specification of fate in mouse ES cells (Niwa et al., 2000). Moreover, reduced Oct4 levels (~50%) promote homogenous Nanog expression and robust pluripotency (Violetta Karwacki-Neisius, PhD thesis UoE). Therefore, it is of interest to determine the expression dynamics of Oct4 in self-renewing ES cells. In order to achieve this, ES cells that contain a GFP transgene driven by the entire regulatory region of *Oct4* (Yeom et al., 1996) were analysed using a similar experimental setup (Figure 3.12). Oct4GiP ES cells cultured at low density in GMEMβ/FCS/LIF alone or supplemented with a MEK inhibitor (PD0325901) showed a moderate change in the CV of *Oct4*:GFP (Figure 3.12A). Notably, GSK3 inhibition (CHIR99021) in Oct4GiP ES cells promotes a reduction in the CV of *Oct4*:GFP (Figure 3.12A). Under all culture conditions tested, the median of *Oct4*:GFP showed a gradual decrease with time (Figure 3.12B).

Interestingly, SONG ES cells show a broader dispersion of *Nanog*:GFP when compared to TNG and FNG ES cells (Figure 3.10). Moreover, SONG ES cells displayed a higher CV of *Nanog*:GFP than TNG and FNG ES cells cultured in GMEMβ/FCS/LIF (Figure 3.11; 3.13A). Culture of TNG and SONG ES cells in the combined presence of CHIR99021 and PD0325901 significantly reduced the CV of *Nanog*:GFP (Figure 3.11A, B). Dual inhibition of GSK3 (CHIR99021)/MEK (PD0325901) in FNG ES cells did not have a significant effect on the CV of *Nanog*:GFP (Figure 3.13A). Strikingly, the CV of *Nanog*:GFP in TNG, SONG and FNG is comparable when the cells are cultured in the presence of N2B27/2i/LIF (Figure 3.13A). Propagation of Oct4GiP ES cells in 2i resulted in a moderate decrease in the CV of *Oct4*:GFP (Figure 3.13A). The median of *Nanog*:GFP was higher in FNG ES cells when compared to TNG and SONG ES cells (Figure 3.13B). *Nanog*:GFP levels were augmented in TNG and SONG ES cells by dual inhibition of GSK3 (CHIR99021)/MEK (PD0325901) (Figure 3.13B). Conversely, Oct4 levels are lower in cells cultured in the presence of GSK3 (CHIR99021) and MEK (PD0325901) inhibitors (Figure 3.13B).

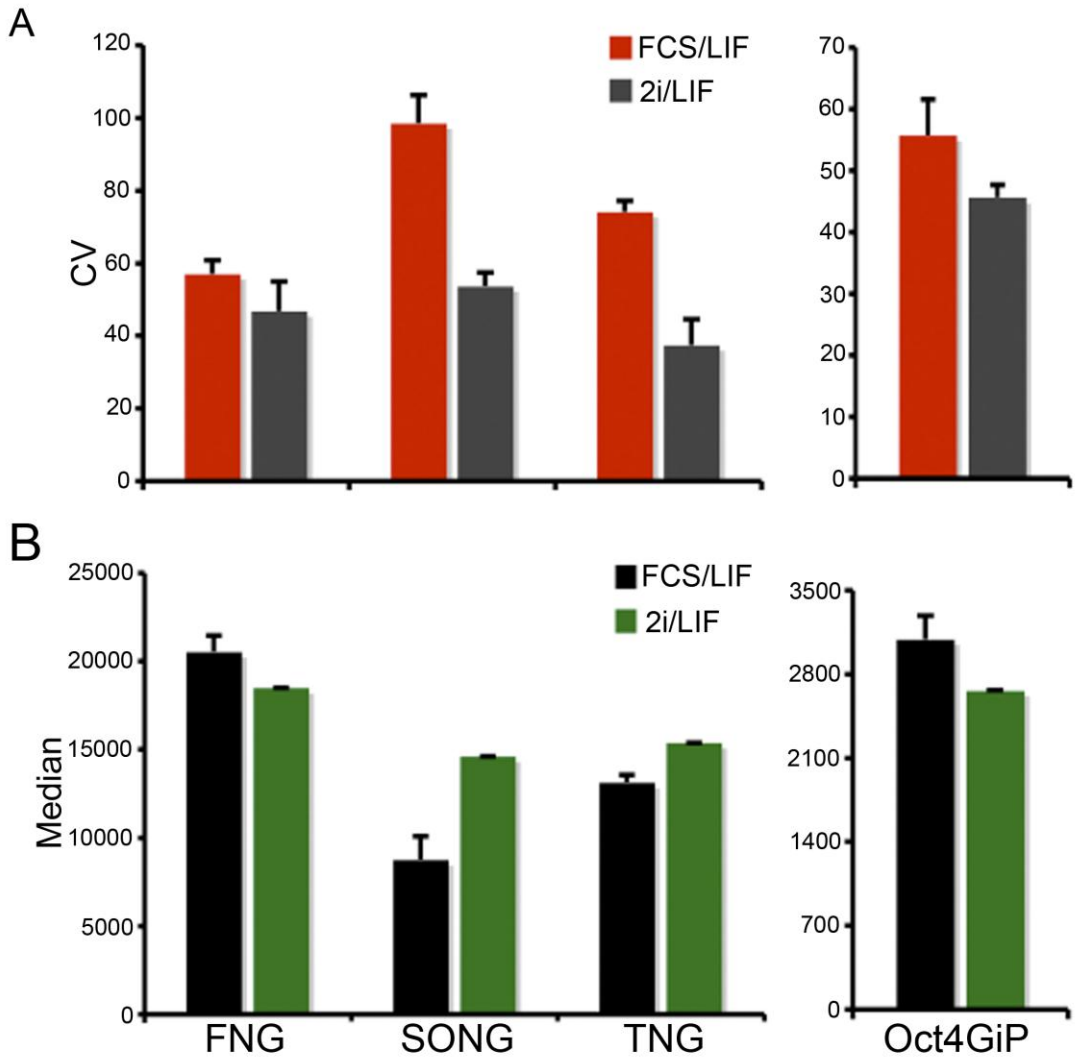


Figure 3. 13 Effect of combined Gsk3 and MEK inhibition on *Nanog*:GFP and *Oct4*:GFP in different mutant ES cells lines.

(A) CV comparison of *Nanog*:GFP and *Oct4*:GFP in mutant ES cells cultured in FCS/LIF and 2i/LIF.

(B) Differences in the median of *Nanog*:GFP and *Oct4*:GFP in mutant ES cells cultured in FCS/LIF and 2i/LIF.

3.5 Discussion

Mouse ES cells are routinely cultured in GMEMβ/FCS/LIF (Smith, 1991). Although this culture condition promotes robust self-renewal of ES cells, differentiation is not completely abolished. Culturing ES cells in the above medium promotes the propagation of a heterogeneous population. Pluripotent transcription factors such as Nanog are heterogeneously expressed (Chambers et al., 2007). Moreover, lineage specific genes are expressed at low levels, a phenomenon termed as lineage priming (Hu et al., 1997; Mikkelsen et al., 2007). In this chapter the role of FGF signaling and Sox2 levels on heterogeneous Nanog expression in mouse ES cells is examined.

3.5.1 The role of Sox2 in ES cell self-renewal and Nanog heterogeneity

Sox2$^{Bgeo/+}$ and *Sox2*$^{Bgeo/flx}$:CreER^{T2} mutant ES cells were used to study the effect of Sox2 levels on ES cell self-renewal and Nanog heterogeneity. Firstly, Sox2 mutant ES cells were characterized. Consistent with a previous report (Masui et al., 2007), deletion of the loxP-flanked Sox2 allele from *Sox2*$^{Bgeo/flx}$:CreER^{T2} ES cells induced differentiation (see Section 3.2). Biochemical analysis of *Sox2*$^{Bgeo/flx}$:CreER^{T2} ES cells following tamoxifen treatment showed disappearance of Sox2 mRNA within 3h, whereas the protein was downregulated between 9-12h (see Section 3.2). This suggests that in undifferentiated ES cells Sox2 is intrinsically a stable protein, increasing the likelihood that Sox2 may be significantly regulated at the protein level. Additionally, one could predict that in response to differentiation cues, the stability of Sox2 may change. Interestingly, although Oct4 expression is maintained

for at least 24h after Cre-mediated deletion of *Sox2*, changes in the expression of *Nr5a2*, *Klf2*, *Esrrb* and *Nanog* can be detected at earlier time points (see Section 3.2). This suggests that Sox2 may have other functions in ES cells than simply stabilizing Oct4 expression as has been proposed (Masui et al., 2007). Future work will be critical to identify Sox2 target genes that are particularly susceptible to changes in Sox2 levels and their effect on pluripotency and ES cell self-renewal. Interestingly, changes in Oct4 protein are apparent prior to transcriptional modulation of *Oct4* after genetic deletion of Sox2 (see section 3.2). This could be an indication that Sox2 may regulate the stability of Oct4 protein, In this regard, $Sox2^{-/-}$ ES cells rescued with a mutant forms of Oct4 may provide useful a tool to further characterize the Oct4-Sox2 interaction.

In post-implantation embryos Sox2 is expressed in the epiblast and extra-embryonic ectoderm, while, at the onset of gastrulation, it becomes restricted to the prospective neural plate and chorion (Wood and Episkopou, 1999). Despite the expression pattern of Sox2 being well documented, its function in the post-implantation epiblast remains poorly characterized. Nonetheless, it is essential as Sox2 deletion in EpiSCs induces overt differentiation into mesenchymal-like cells (see Section 3.3). One possible role for Sox2 in the post-implantation epiblast is to promote the expression of Oct4, as has been suggested to occur in ES cells. The mechanism through which this would be achieved is not clear. It has been suggested that the induction of Oct4 expression may occur through the Sox2 target gene, Nr5a2. Indeed, Nr5a2 has been shown to bind to Oct4 proximal promoter and proximal enhancer (Gu et al., 2005). Moreover, Nr5a2 deficient embryos displayed disorganisation of the egg cylinder and premature loss of Oct4 expression (Gu et al.,

2005). However, chimaeras produced by tetraploid aggregation of Nr5a2$^{-/-}$ cells were able to develop through gastrulation, suggesting that the loss of Oct4 expression in the epiblast is likely to be the result of the defective formation of the visceral endoderm (Labelle-Dumais et al., 2006). Furthermore, Nr5a2 can be deleted from ES cells without an apparent effect on self-renewal (Gu et al., 2005). Additionally, Oct4 is abundant in EpiSC, whereas the Nr5a nuclear receptors are not expressed (see Chapter 5; (Guo and Smith, 2010)). Therefore, Nr5a2 is not essential for the expression of Oct4 in the epiblast or in its *in vitro* derivatives. Further characterization is necessary to determine the mechanism through which Sox2 promotes Oct4 expression and additional functions that Sox2 may have in pluripotent cells.

Recent work has shown that changes in Oct4 levels have a profound effect on ES cell self-renewal. Oct4 heterozygote ES cells are considerably less heterogeneous in Nanog expression than wildtype ES cells. Consistent with previous results, these cells show a bias towards self-renewal and less readily differentiate (Violetta Karwacki-Neisius, PhD thesis UoE). Furthermore, it has been shown that Oct4 and Sox2 bind their motifs independently (Ambrosetti et al., 1997; Ambrosetti et al., 2000), however, their binding to DNA is stabilized by protein-protein interaction in the ternary complex (Remenyi et al., 2003). Due to the close functional relationship between Oct4 and Sox2 it is feasible that the pluripotency network is susceptible to variations in Sox2 levels. Sox2$^{Bgeo/+}$ ES cells were used to study the effect of lower Sox2 levels on Nanog heterogeneity. A Nanog reporter line was made by introducing eGFP at the *Nanog* AUG codon of *Sox2$^{Bgeo/+}$* ES cells (SONG). Side-by-side experiments demonstrated that cells with reduced Sox2 levels

(SONG) displayed greater heterogeneity for Nanog expression than cells with wild-type Sox2 levels (see Section 3.4). It is important to mention that the observed variation in Nanog levels were not the result of spontaneous differentiation, but rather occurred in the undifferentiated ($Oct4^{+}$/SSEA-1^{+}) fraction of SONG ES cells. This would be consistent with a strictly positive role for Sox2 on Nanog expression, either through the proximal Oct4/Sox2 site (Kuroda et al., 2005; Rodda et al., 2005) or through binding to the -5kb regulatory element (Loh et al., 2006). Moreover, lower Sox2 levels have been shown to be permissive for hES cell and EpiSC self-renewal (Han et al., 2010; Osorno et al., 2012; Wang et al., 2012). As EpiSCs display lower Sox2 levels when compared to ES cells, it would be interesting to determine whether, similar to Nanog, Sox2 downregulation is required to allow the transition from naïve to primed pluripotency. In support of this hypothesis, it has been shown that Sox2 knockdown leads to upregulation of epiblast and trophectodermal markers (Ivanova et al., 2006). The effect of lower Sox2 levels on Nanog heterogeneity contrasts markedly to those observed for $Oct4^{+/-}$ ES cells (see Section 3.4; Violetta Karwacki-Neisius, PhD thesis UoE), suggesting that changes in Oct4 levels have a blatant effect on the expression of pluripotency network components, whereas variation in Sox2 levels may have more subtle effects. Future studies will be required to reveal the precise function of the Sox2/Oct4 and Sox2/Nanog interactions in the context of the GRN of pluripotent cells.

Intriguing is the observation that MEK (PD0325901) and GSK3 (CHIR99021) inhibition in $Sox2^{Bgeo/+}$ ES cells greatly reduces Nanog heterogeneity (see Section 3.4). This suggests that changes in the pluripotency network induced by reduction in Sox2 levels may be mediated by MEK and GSK3 signaling. Interestingly, it has

been reported that Sox2 knockdown in ES cells results in ERK1/2 hyper-phosphorylation (Ivanova et al., 2006), suggesting that Sox2 may be necessary to moderate MEK/ERK activation in ES cells. In summary, changes in Sox2 levels have a more modest effect on Nanog heterogeneity than variation of Oct4 levels. Further studies are necessary to determine whether ES cells with reduced Sox2 levels show hyper-activation of ERK1/2 and GSK3, supporting the role of Sox2 in the stabilization of naïve pluripotency.

3.5.2 Role of FGF signaling on Nanog heterogeneity

There is a growing body of information strongly supporting the relation between FGF/MEK/ERK signaling pathway and the promotion of differentiation (Burdon et al., 1999; Kunath et al., 2007; Stavridis et al., 2007). Moreover, *in vivo* studies demonstrating that FGF/MEK/ERK activity is essential for the formation of the lineages present in the ICM provides confidence that such pathway is not an artefact of the *in vitro* model (Chazaud et al., 2006; Nichols et al., 2009; Yamanaka et al., 2010). As a result, the observation that inhibition of FGF/MEK/ERK pathway *in vivo* promotes bias toward the formation of Nanog^{high} cells (Nichols et al., 2009) is relevant in the context of ES cell biology.

In order to study the role of FGF signaling on *Nanog* expression, a reporter line was made by introducing eGFP into the *Nanog* AUG codon of *FGF4*$^{-/-}$ ES cells (FNG). Side-by-side experiment using FNG and TNG ES cells clearly showed that FGF4$^{-/-}$ ES cells are less heterogeneous for Nanog than ES cells with a functional *FGF4*. Strikingly, supplementation of exogenous bFGF restored the differentiation

capacity of FNG ES cells by re-instating heterogeneous Nanog expression. However, ES cells with functional FGF4 did not display increased differentiation upon addition of exogenous bFGF, further supporting the notion that FGF signaling in ES cells is necessary to prime the cells for differentiation. Interestingly, addition of exogenous FGF to wildtype ES cells did not produce a significant increase in Nanog heterogeneity, suggesting that FGF signaling may have a saturation point. One can predict that basal FGF activation may be necessary to promote early differentiation events, inducing lineage priming, but the pathway saturates promptly preventing it from acting in the final stages of differentiation. Alternatively, it is possible that FGF signaling is required to generate a balanced $Nanog^{high}$/$Nanog^{low}$ population, with other signaling pathways needed to promote commitment.

Chapter 4

The developmental loss of somatic pluripotency is reversed by ectopic Oct4

4.1 Introduction

The original studies that proved that the embryo proper contained pluripotent cells were done by testing their ability to form teratocarcinoma (Solter et al., 1970; Stevens, 1968, 1970). Subsequent analysis showed that the cellular origin of the teratocarcinoma forming potential was the epiblast (Diwan and Stevens, 1976). The pluripotent epiblast first appears in the ICM of mouse blastocysts. After implantation the epiblast undergoes dramatic expansion and morphogenesis, resulting in the transformation of the epiblast from an unstructured cell mass into a columnar epithelium.

Previous analyses have demonstrated that somatic pluripotency is lost in the mouse at some point between E7.5 and E8.5 (Damjanov et al., 1971). Moreover, pluripotent stem cells have been derived from the epiblast layer of post-implantation mouse embryos from E5.5-6.5 (Bao et al., 2009; Brons et al., 2007; Greber et al., 2010; Tesar et al., 2007). It is well documented that pluripotency in pre-implantation embryos and ES cells is governed by a gene regulatory network centred on the transcription factors Oct4, Sox2 and Nanog (Avilion et al., 2003;

Chambers et al., 2007; Masui et al., 2007; Mitsui et al., 2003; Nichols et al., 1998; Niwa et al., 2000; Silva et al., 2009). Notably, these factors are also expressed in the post-implantation epiblast and its *in vitro* derivative EpiSC (Avilion et al., 2003; Brons et al., 2007; Downs, 2008; Hart et al., 2004; Scholer et al., 1990a; Tesar et al., 2007; Wood and Episkopou, 1999; Yeom et al., 1996). Interestingly, the expression of Nanog, Oct4 and Sox2 in post-implantation embryos does not precisely match the reported presence of pluripotent cells. Indeed, Sox2 is expressed in the neurectoderm beyond the onset of somitogenesis (Avilion et al., 2003; Wood and Episkopou, 1999). Oct4 can be detected in somatic cells until about ~E8.5, thereafter it becomes restricted to the primordial germ cells (Downs, 2008; Scholer et al., 1990a; Scholer et al., 1990b; Yeom et al., 1996). Nanog disappears from somatic cells at some point between E7.5-8.5 (Hart et al., 2004; Hatano et al., 2005). It is therefore of considerable interest to determine the expression of these key factors in the post-implantation embryos, and define their role in the maintenance of primed pluripotency.

4.2 Pluripotency disappears from the epiblast as Oct4 and Nanog levels decline

The expression of Oct4, Sox2 and Nanog in the post-implantation embryo was analysed. Immunofluorescence, in situ hybridization and GFP reporter expression from the endogenous *Nanog* gene, indicate that from the start of gastrulation, Nanog is strongly expressed in the proximal-posterior epiblast and

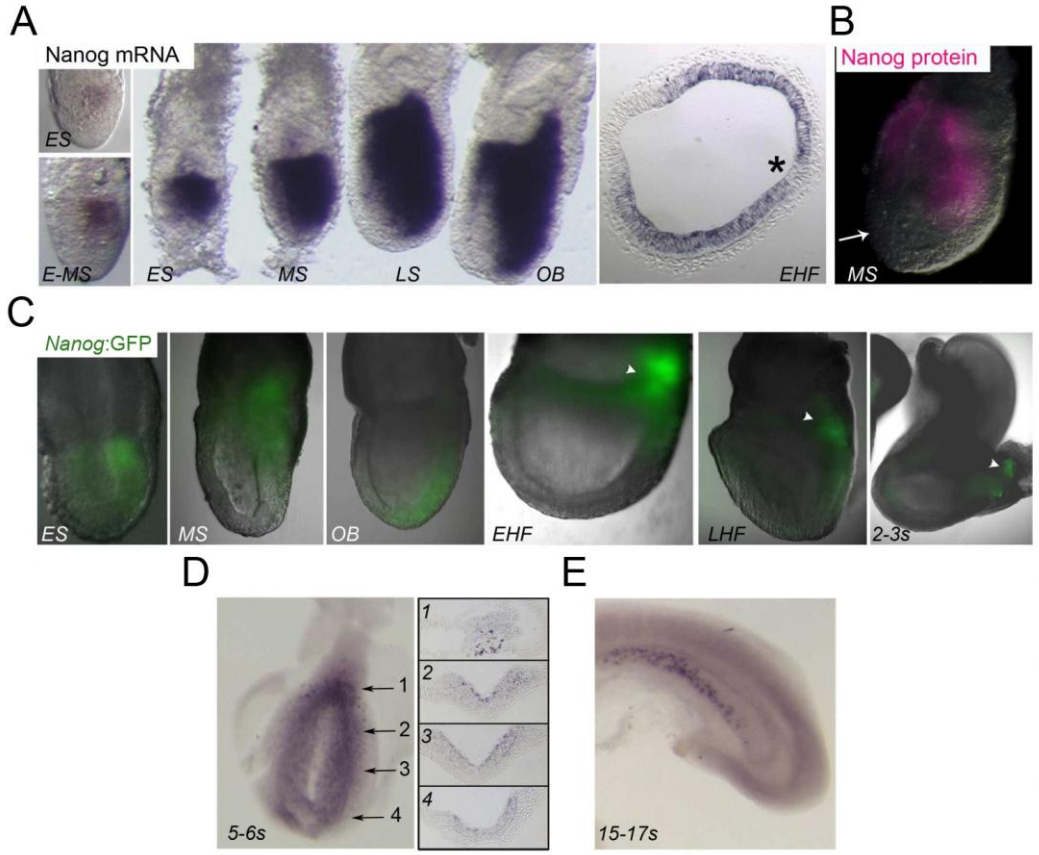


Figure 4. 1 Expression of Nanog mRNA, protein and Nanog:GFP in post-implantation embryos.

(A) Whole-mount in situ hybridization in ES to EHF embryos for *Nanog*. Pre-somitogenesis embryos were staged according to Downs and Davies classification (Downs and Davies, 1993). Older embryos were staged by number of somite pairs. Asterisk, primitive streak in transverse section of EHF stage embryo showing cells downregulating Nanog mRNA as they ingress into the primitive streak. ES, early streak; MS, mid-streak; LS, late streak; OB, no allantoic bud; EHF, early headfold; LHF, late headfold.

(B) NANOG localisation in a MS stage embryo. Arrow, distal-anterior region lacking Nanog expression.

(C) *Nanog*:GFP localisation in ES to 2-3s stage embryos. Arrowheads, PGCs expressing *Nanog*:GFP.

(D) Wholemount in situ hybridization for Oct4 in 5-6s stage embryo. Left: whole embryo. Right: transverse sections showing expression in PGCs(1) and somatic tissue (2-4).

(E) Wholemount in situ hybridization for Oct4 in 15-17s stage embryo showing expression in PGCs.

undetectable in the distal-anterior epiblast (Figure 4.1A-C). It was also observed that cells downregulate Nanog mRNA as they ingress into the primitive streak (Figure 4.1A). Nanog disappears completely from the epiblast at the onset of somitogenesis, and is thereafter expressed exclusively in the germ line (Figure 4.1A-C). On the other hand, Oct4 is expressed in the entire epiblast until the late bud stage and its expression persists in somatic cells until about 12-15 somite (s) embryos (Figure 4.1D-E) (Downs, 2008; Scholer et al., 1990a; Yeom et al., 1996). After ~12-15s stage, Oct4 is exclusively expressed in the germ line (Figure 4.1E). Quantitive RT-PCR (qPCR) was used to analyse the expression of the core pluripotency transcription factors during the time window in which somatic pluripotency disappears (Damjanov et al., 1971)). Sox2 expression gradually increases, while Nanog and Oct4 decline between E7.5-E8.5, eventually becoming undetectable at 3-5s and 12 15s respectively (Figure 4.2A).

4.3 Pluripotency disappears at the onset of somitogenesis and is equivalent in Nanog-negative and Nanog-positive epiblast

Given the observation that Nanog expression showed the closest correlation with pluripotent cells in implantation embryos, the potency of Nanog expressing and non-expressing cells was tested. The last detectable expression of *Nanog*:GFP in somatic cells is the distal tip at 0-1s (Figure 4.2B). Furthermore, the distal region of 2-5s retained Oct4 expression (Figure 4.3A). Indeed, qPCR analysis of the distal tip of 2-5s embryos revealed expression of Oct4 and Sox2, but confirmed that Nanog

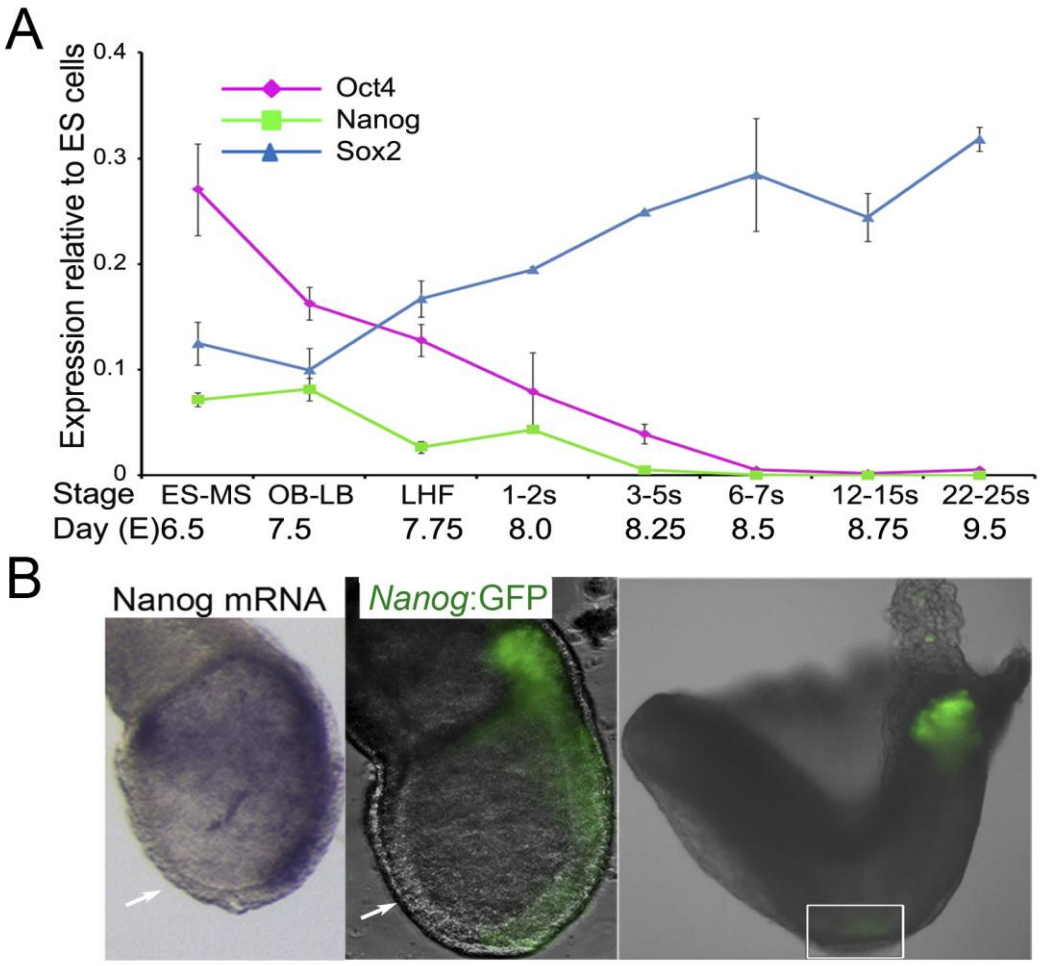


Figure 4. 2 Oct4 and Nanog levels decline shortly after the onset of somitogenesis.

(A) qPCR analysis of Oct4, Nanog and Sox2 mRNAs in pooled embryos. Error bars: s.e.m. of 3 biological replicates. mRNA preparations and qPCRs were performed by Dr. Anestis Tsakiridis.

(B) Nanog mRNA and *Nanog*:GFP expression. Arrow, distal-anterior region lacking Nanog expression. Box, distal region showing residual *Nanog*:GFP expression.

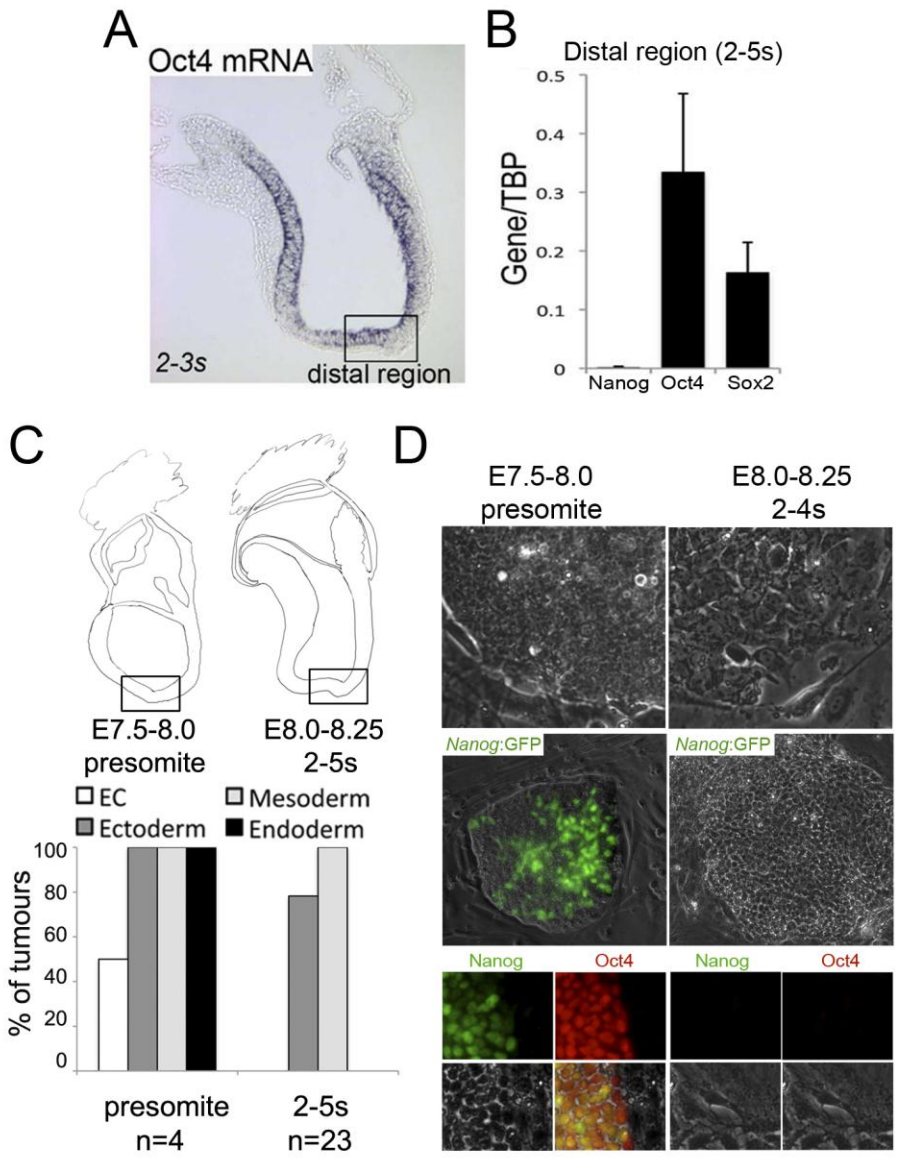


Figure 4. 3 Pluripotency disappears from the epiblast as Oct4 and Nanog levels decline.

(A) Sagittal section of a 2-3s stage embryo analysed by in situ hybridization for *Oct4*.

(B) qPCR of distal region in 2-5s embryos for Nanog, Oct4 and Sox2. Values normalized to TBP and expressed relative to ES cell levels.

(C) Teratocarcinoma-forming capacity of distal regions of pre-somite and somitogenesis stage embryos (boxes).

(D) Ability of E7.5-8.25 embryos to form EpiSC. Top: EpiSC colony morphology. Middle: Expression of *Nanog*:GFP. Bottom: Immunoreactivity to Nanog and Oct4. pluripotency disappears at early somitogenesis, coincident with extinction of Nanog but before Oct4 becomes undetectable.

transcripts were absent (Figure 4.3B). Therefore, the distal region of headfold and 2-5s embryos was tested for pluripotency (Figure 4.3C-D). Consistent with published data, kidney capsule grafts of headfold stage embryos produced large teratocarcinomas (Figure 4.3C) (Beddington, 1983). Conversely, similar tissue from 2-5s embryos produced small growths devoid of EC cells and endoderm (Figure 4.3C).

Next, the concordance of EpiSC derivation and teratocarcinoma formation was tested. EpiSC lines have previously been derived from E5.5-6.5 (Bao et al., 2009; Brons et al., 2007; Greber et al., 2010; Tesar et al., 2007). Given the observation that Oct4, Sox2 and Nanog are expressed in headfold stage embryos their ability to generate EpiSC was tested. Upon explantation of tissue from E7.5-E8.0 embryos onto EpiSC culture conditions, *Nanog*:GFP was readily detected in explants (Figure 4.3D). These explants could be passaged and in all cases (8/8) gave rise to EpiSC lines that expressed Oct4 and Nanog protein (Figure 4.3D). In contrast, explants from 2-4s embryos did not express *Nanog*:GFP (0/6). Upon passaging, these explants produced morphologically differentiated cells that did not express Oct4 or Nanog (Figure 4.3D). Thus, EpiSC derivation ability is an effective assay to test the presence of primed pluripotency in post-implantation embryos.

To examine the relationship between pluripotency and Nanog expression in more detail, a staged series of *Nanog*:GFP embryos was isolated, separated into Nanog-nonexpressing (distal/anterior) and Nanog-expressing (proximal/posterior) regions, and explanted in EpiSC culture conditions (Figure 4.4A). The presence of

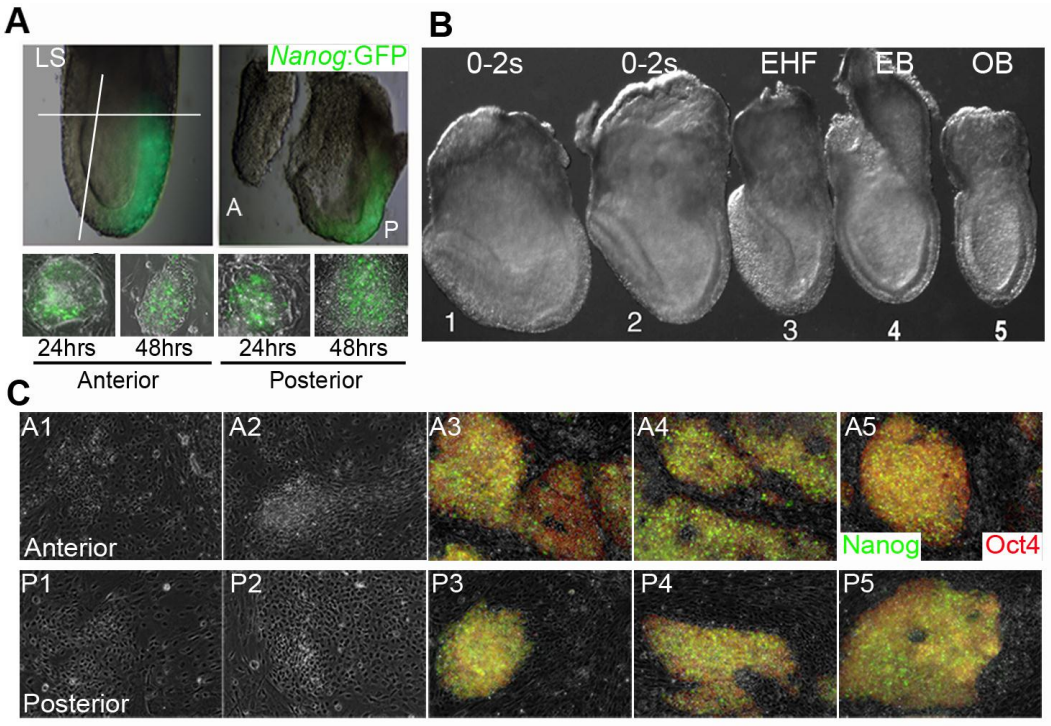


Figure 4. 4 Pluripotency disappears at the onset of somitogenesis and is equivalent in Nanog-negative and Nanog-positive epiblast.

(A) E7.5 embryos expressing *Nanog*:GFP were dissected along the indicated lines into anterior (A) and posterior (P) fragments. Bottom; *Nanog*:GFP expression upon explantation onto feeders and DMEM-F12β□KOSR/Activin/bFGF.

(B) Embryos before micro-dissection, arranged from oldest (left) to youngest (right).

(C) Immuno-histochemical detection of Oct4 and Nanog in representative colonies of EpiSC lines derived from these embryos. A1-5 and P1-5 correspond to lines derived from anterior and posterior fragments of numbered embryos shown in panel B.

bright *Nanog*:GFP$^+$ cells at the base of the allantois allowed us to confirm exclusion of these cells from posterior explants. Without exception, prior to somitogenesis, anterior and posterior explants showed Nanog expression at 24-48 hours (Figure 4.4A) and primary explants displayed robust Oct4 and Nanog expression (Figure 4.4B-C). Moreover, EpiSC lines were readily derived from anterior and posterior regions of pre-somitogenesis embryos (n=12 for each; Figure 4.5A-B). Interestingly, explants from somitogenesis stage embryos failed to reactivate Nanog expression and did not generate EpiSC lines (0/23; Figure 4.3D; Figure 4.4B-C). Additionally, the potency of Nanog-nonexpressing (distal/anterior) and Nanog-expressing (proximal/posterior) regions was assessed using kidney capsule grafts. Notably, anterior and posterior regions showed equivalent teratocarcinoma-forming capacity (Figure 4.6). In summary, somatic pluripotency disappears between headfold and somitogenesis stages. Moreover, prior to somitogenesis, Nanog-expressing and Nanog-nonexpressing regions remain pluripotent. However, the observation that Nanog is re-expressed in anterior explants within 24 hours of culture in EpiSC conditions, suggests that the epiblast shows plasticity in gene expression.

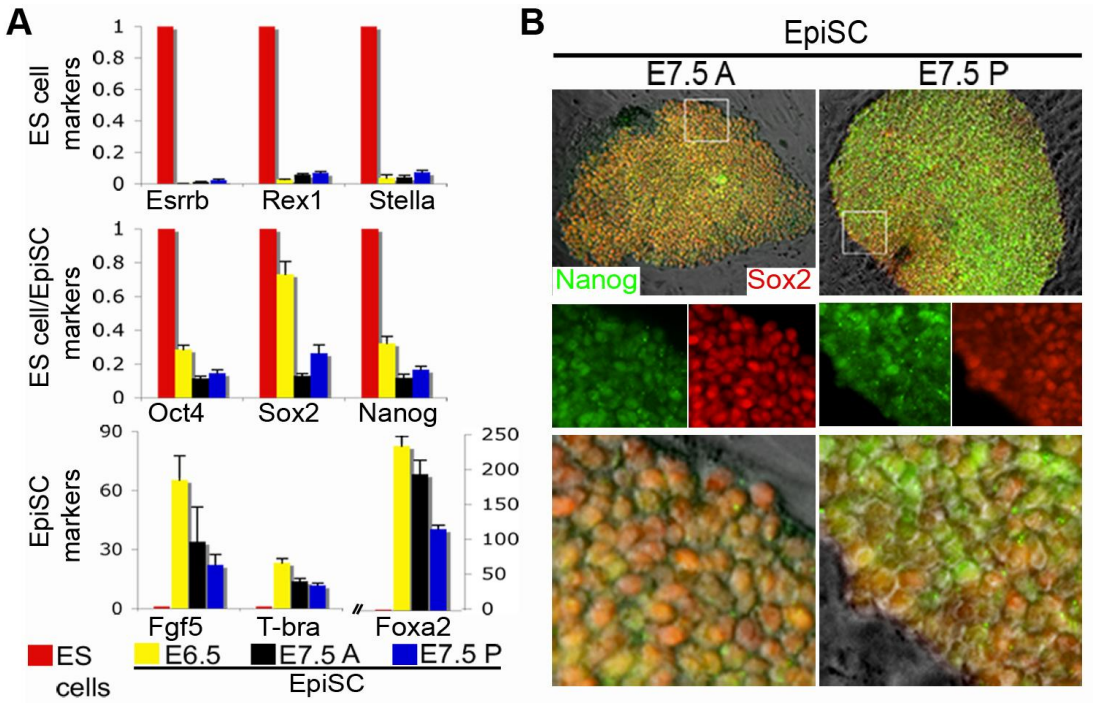


Figure 4. 5 Nanog-negative and Nanog-positive epiblast regions readily make EpiSC.

(A) qPCR analysis of representative anterior and posterior E7.5 EpiSC lines. ES cells and E6.5 EpiSC were used as controls. ES cell/EpiSC markers are common to both ES and EpiSC lines.

(B) Nanog and Sox2 immunofluorescence in representative anterior and posterior EpiSC lines. Box in top panels: region magnified below.

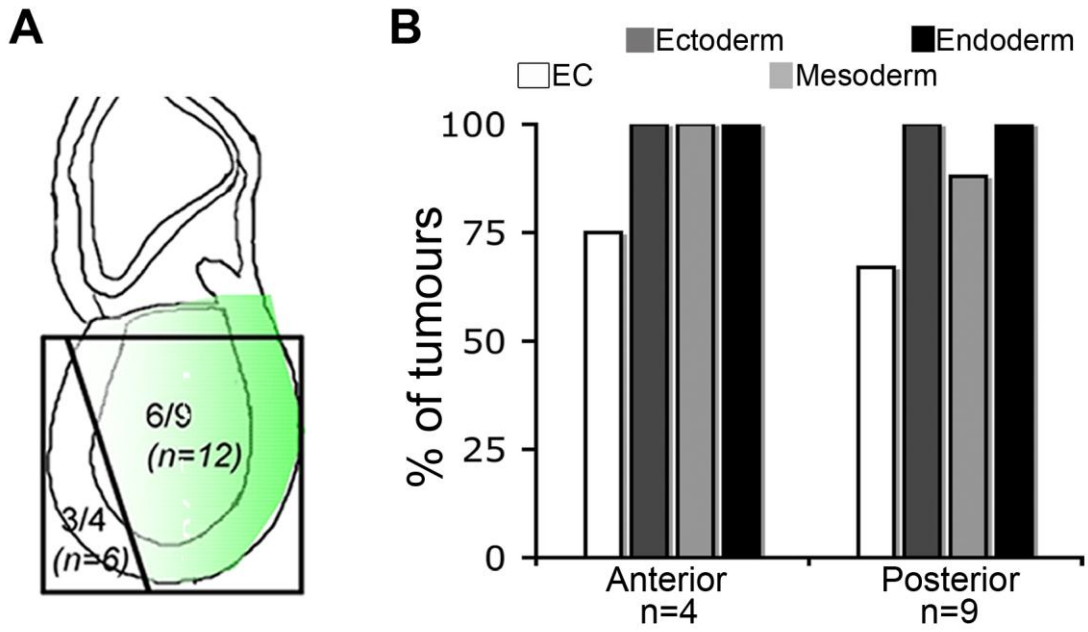


Figure 4. 6 Anterior and posterior epiblast at E7.5 are pluripotent.

(A) Nanog:GFP embryos were dissected into Nanog:GFP^{+} (posterior) and Nanog:GFP^{-} (anterior) regions (as depicted in Figure 4.3A). The extra-embryonic region was removed, as well as the most proximal epiblast containing the PGCs. The embryonic fragments were checked with fluorescence optics to verify the accuracy of the dissection. Kidney capsule grafts were performed as described previously (Tam, 1990). The number of teratocarcinomas in anterior and posterior regions is depicted as a fraction of total tumours (n, number of grafts performed).

(B) Anterior/posterior-derived tumour composition. Grafts shown in (A) were scored for germ layer differentiation. The presence or absence of EC cells and germ layer representative cells/structures was scored for whole tumours.

4.4 Nanog is not required for post-implantation pluripotency

At least two possibilities may explain the observation that Nanog-nonexpressing epiblast cells are pluripotent: (1) these cells represent a latent pluripotent state that requires Nanog reactivation to manifest pluripotency, or (2) Nanog is not essential for epiblast pluripotency. In order to distinguish between these possibilities, ES cells genetically depleted for Nanog were used to produce E6.5-E7.5 chimaeras, which were then tested for their ability to generate EpiSCs (Figure 4.7). Two *Nanog*$^{-/-}$ cell lines TβC44cre6 and RCNβH-B(t), alongside a control derivative line RCNβH-B(t)R, in which one of the non-functional Nanog alleles has been repaired by homologous recombination, were used for these experiments (Chambers et al., 2007). Discrimination of wild-type and ES-derived cells is possible because RCNβH-B(t) and RCNβH-B(t)R express GFP constitutively, whereas TβC44cre6 expresses GFP from a targeted Nanog-null allele. *Nanog*$^{-/-}$ cells formed chimaeras indistinguishable from wild-type or *Nanog*$^{-/+}$ cells (Figure 4.7B). Primary explants from *Nanog*$^{-/-}$ chimaeras were picked and expanded to produce two independent EpiSC lines that lacked Nanog, but showed robust Oct4 and Sox2 expression (Figure 4.7B). To exclude the formal possibility that derivation or survival of chimaera-derived Nanog-null EpiSC required co-culture with wild-type cells, pure *Nanog*$^{-/-}$ ES cell populations were differentiated *in vitro* into EpiSC (Guo et al., 2009). Two independent *in vitro* derived *Nanog*$^{-/-}$ EpiSC lines were readily obtained (Figure 4.8). *In vitro* derived TβC44cre6 and RCNβH-B(t) displayed robust Oct4 and Sox2 expression (Figure 4.8A). Furthermore, the two *in vitro* derived *Nanog*$^{-/-}$ EpiSC expressed EpiSC, but did not express ES cell markers (Figure 4.8B).

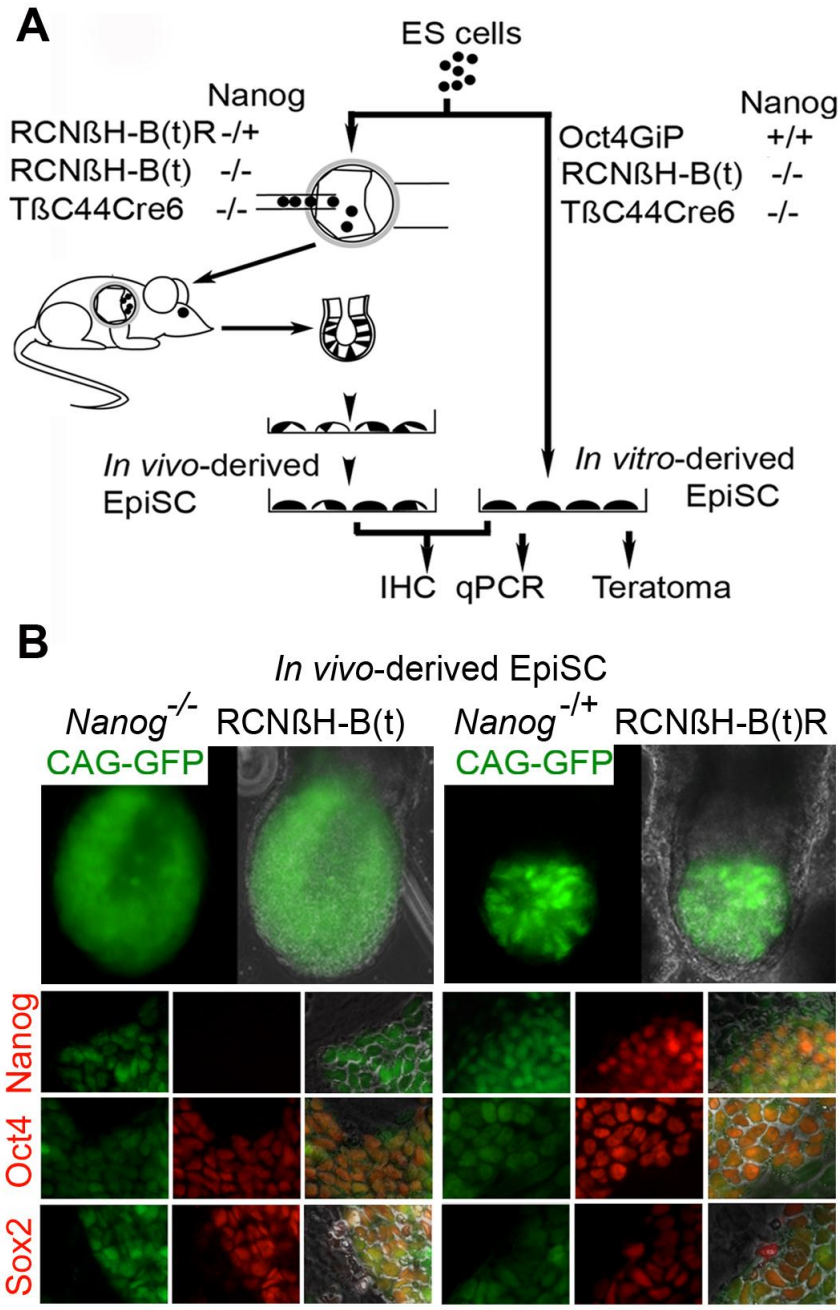


Figure 4. 7 Nanog is dispensable for the derivation and maintenance embryo-derived EpiSC.

(A) Experimental strategies for derivation and analysis of *Nanog*$^{-/-}$ EpiSC lines used throughout the chapter.

(B) Top: chimeric embryos with *Nanog* mutant cells expressing constitutive GFP. Below: Oct4, Sox2 and Nanog immunofluorescence in established EpiSC lines from chimeric embryos. *Nanog*$^{-/-}$ RCNβH-B(t) EpiSC line was analysed alongside the control line *Nanog*$^{-/+}$ RCNβH-B(t)R.

Nanog$^{-/-}$ EpiSCs, alongside a control *Nanog*$^{+/+}$ EpiSCs, were grafted into the kidney capsule of mice. Remarkably, similar to control *Nanog*$^{+/+}$ EpiSC, Nanog$^{-/-}$ EpiSC produced teratocarcinomas (Figure 4.8C-D). Put together these data show that Nanog is required neither for the establishment nor the maintenance of primed pluripotency. This is in stark contrast with the apparent necessity for Nanog in the acquisition of naïve pluripotency (Silva et al., 2009).

It has previously been proposed that Activin promotes self-renewal in hES cells and mouse EpiSC through the activation of Nanog (Greber et al., 2010; Vallier et al., 2009). In mouse embryos, Nanog tracks Nodal expression in the postimplantation epiblast (Figure 4.1; (Brennan et al., 2001)). Moreover, Nodal deletion results in premature loss of the pluripotency factors Oct4 and Nanog (Brennan et al., 2001; Mesnard et al., 2006). Unexpectedly, as shown in this chapter, EpiSCs can be established and maintained without Nanog. To further investigate the signaling requirements involved in the maintenance of primed pluripotency, specific inhibitors for Activin (SB431542) or FGF (PD0325901) signaling pathways were used. In the presence of an Activin inhibitor (SB431542), explanted epiblast tissue lost expression of *Nanog*:GFP (Figure 4.9A). Interestingly, when an MEK inhibitor (PD0325901) was used, no significant effect was on the expression of *Nanog*:GFP (Figure 4.9A). This is consistent with a previous report showing that FGF signaling in EpiSC does not cooperate with Smad2/3 to enhance Nanog expression (Greber et al., 2010). Moreover, similar to *Nanog*$^{+/-}$ explants, *Nanog*$^{-/-}$ EpiSC lines treated with Activin inhibitor (SB431542) failed to maintain the undifferentiated morphology and downregulated *Nanog*:GFP (Figure 4.9B-C). It is intriguing that in the presence of a MEK inhibitor (PD0325901), *Nanog*$^{-/-}$ cells

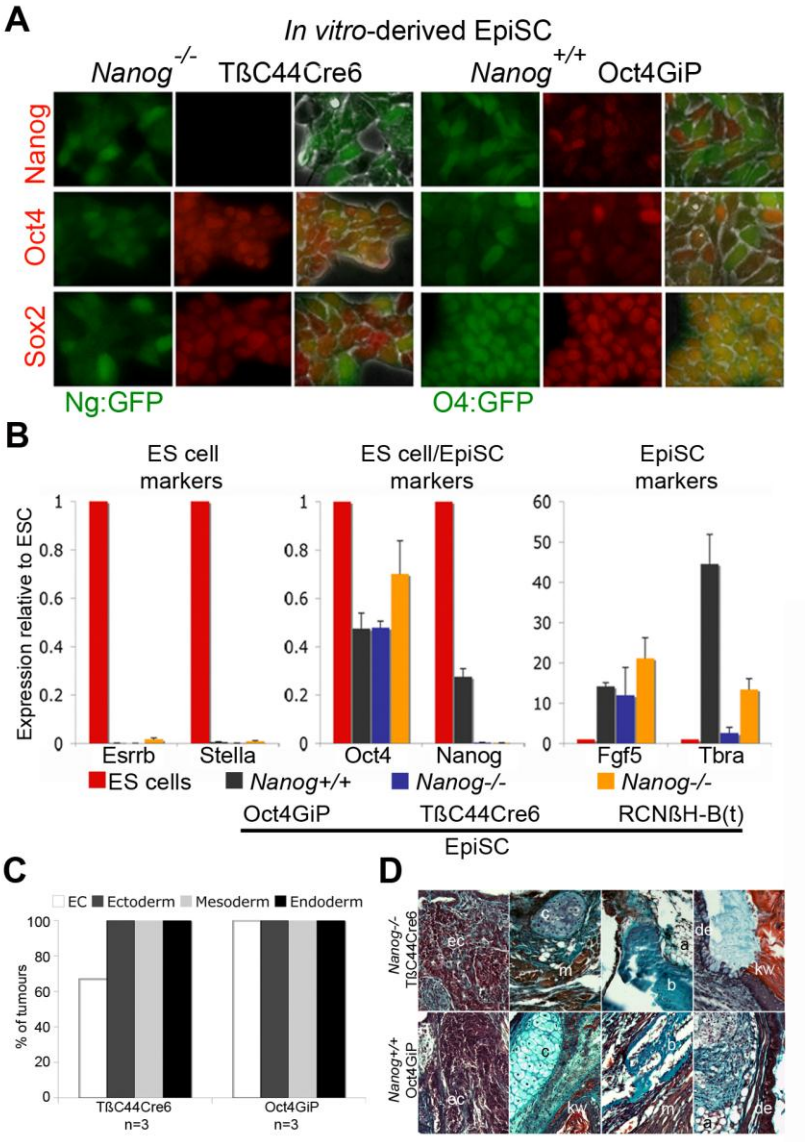


Figure 4. 8 Nanog is dispensable for the derivation and maintenance of EpiSC.

(A) Oct4, Sox2 and Nanog immunofluorescence in TβC44cre6 (*Nanog*$^{-/-}$) and Oct4GiP (*Nanog*$^{+/+}$) EpiSC lines.

(B) qPCR analysis of in ES-derived EpiSC lines. Analyses were determined relative to TATA box-binding protein (TBP) and normalized to ES cells. Results are the average 3 independent RNA preparations.

(C) Scoring of germ layer differentiation of the indicated EpiSC cell lines following a teratocarcinoma-forming assay.

(D) Masson's trichrome-stained tumour sections derived from the indicated EpiSC lines. ec, embryonal carcinoma cells; c, cartilage; m, skeletal muscle; b, bone; a, adipose; de, digestive epithelium; kw, keratin whorl.

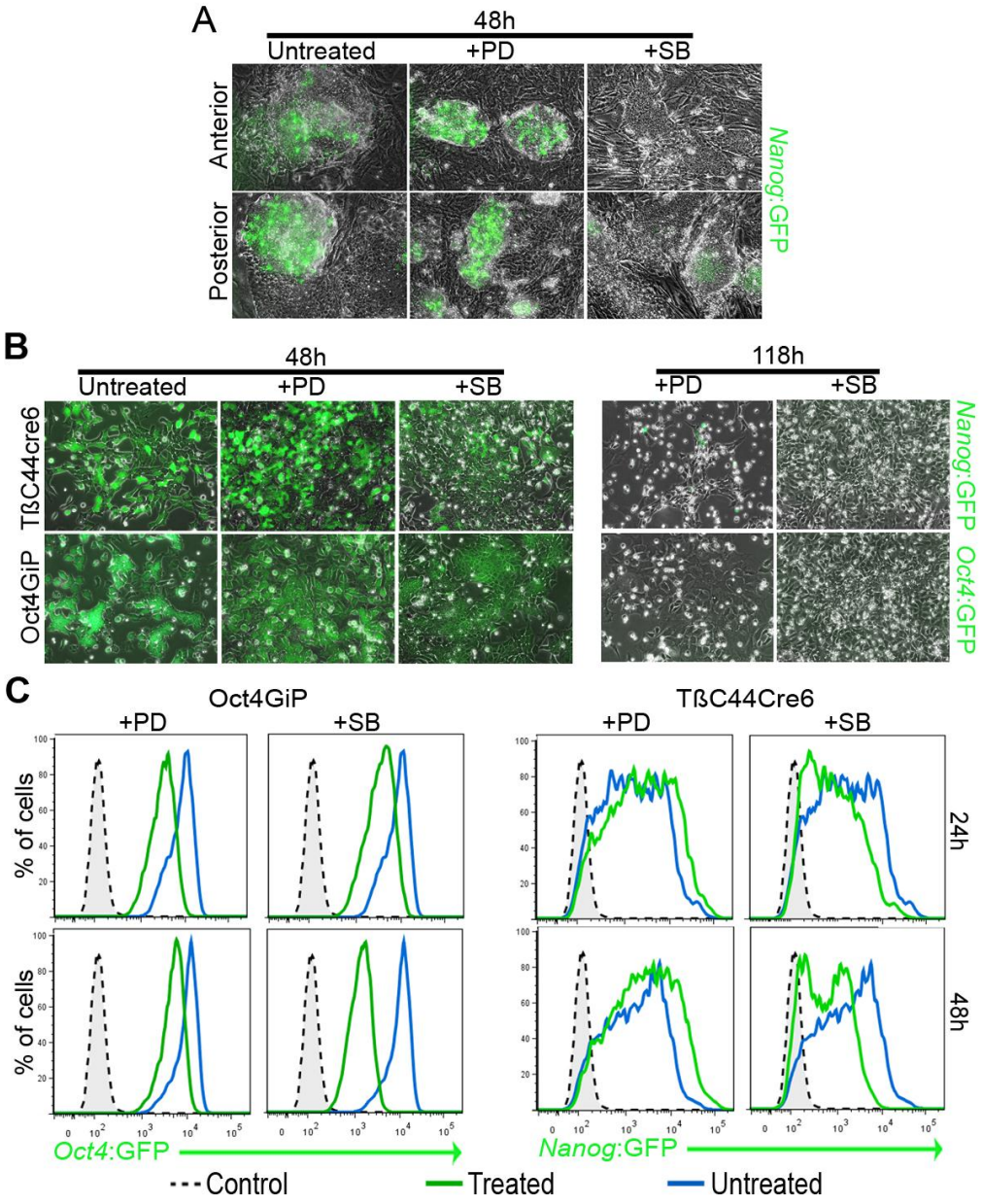


Figure 4. 9 Signal inhibition in primary explants and EpiSC lines.

(A) Primary colonies derived from anterior and posterior fragments of E7.5 embryos 48 hours after explantation showing *Nanog*:GFP expression in 10 μM SB431542 (SB) or 1 μM PD0325901 (PD).

(B) TβC44cre6 (*Nanog*$^{-/GFP}$) and Oct4GiP (*Nanog*$^{+/+}$) EpiSC lines treated with 10 μM SB431542 or 1 μM PD0325901 for 48 hours or 118 hours and examined by fluorescence and bright-field imaging.

(C) Flow cytometry analysis of EpiSC lines examined in B at 24 and 48 hours. Wild type EpiSC were used as controls.

Initially upregulated *Nanog*:GFP expression at 48 hours, but eventually downregulated *Nanog*:GFP expression and EpiSC morphology by 118 hours (Figure 4.9B-C). Together, these results indicate that, in EpiSCs, Nanog is a target of Activin signaling (Greber et al., 2010; Vallier et al., 2009), but is dispensable for Activin-dependent self-renewal.

4.5 Role of Oct4 in post-implantation pluripotency

Based on the observation that primed pluripotency can exist in the absence of Nanog, the role of Oct4 was further analysed in post-implantation epiblast pluripotency. Oct4 is widely expressed in the post-implantation embryos up until the late bud stage (Scholer et al., 1990a; Yeom et al., 1996). However, Oct4 is consistently lower in the anterior part of the embryo from the early headfold stage (Figure 4.10A, C) (Downs, 2008). Comparison of Oct4 mRNA in the prospective forebrain (region 1) and neighbouring tissue (region2), demonstrated that Oct4 mRNA was consistently lower in the anteriomost region (Figure 4.10A-C). Furthermore, Oct4 mRNA declined in both regions with embryonic age (Figure 4.10C). Next, region 1 and 2 were tested for their ability to reactivate Nanog expression in EpiSC culture conditions (Figure 4.10D). Throughout the embryonic stages analysed, region 1 showed a lower efficiency of Nanog reactivation than region 2 (Figure 4.10D). Moreover, the efficiency of Nanog-reactivation in both regions declined with developmental stage (Figure 4.10D). Therefore, similar to ES cells, a specific threshold of Oct4 levels may define pluripotency in the post-implantation epiblast.

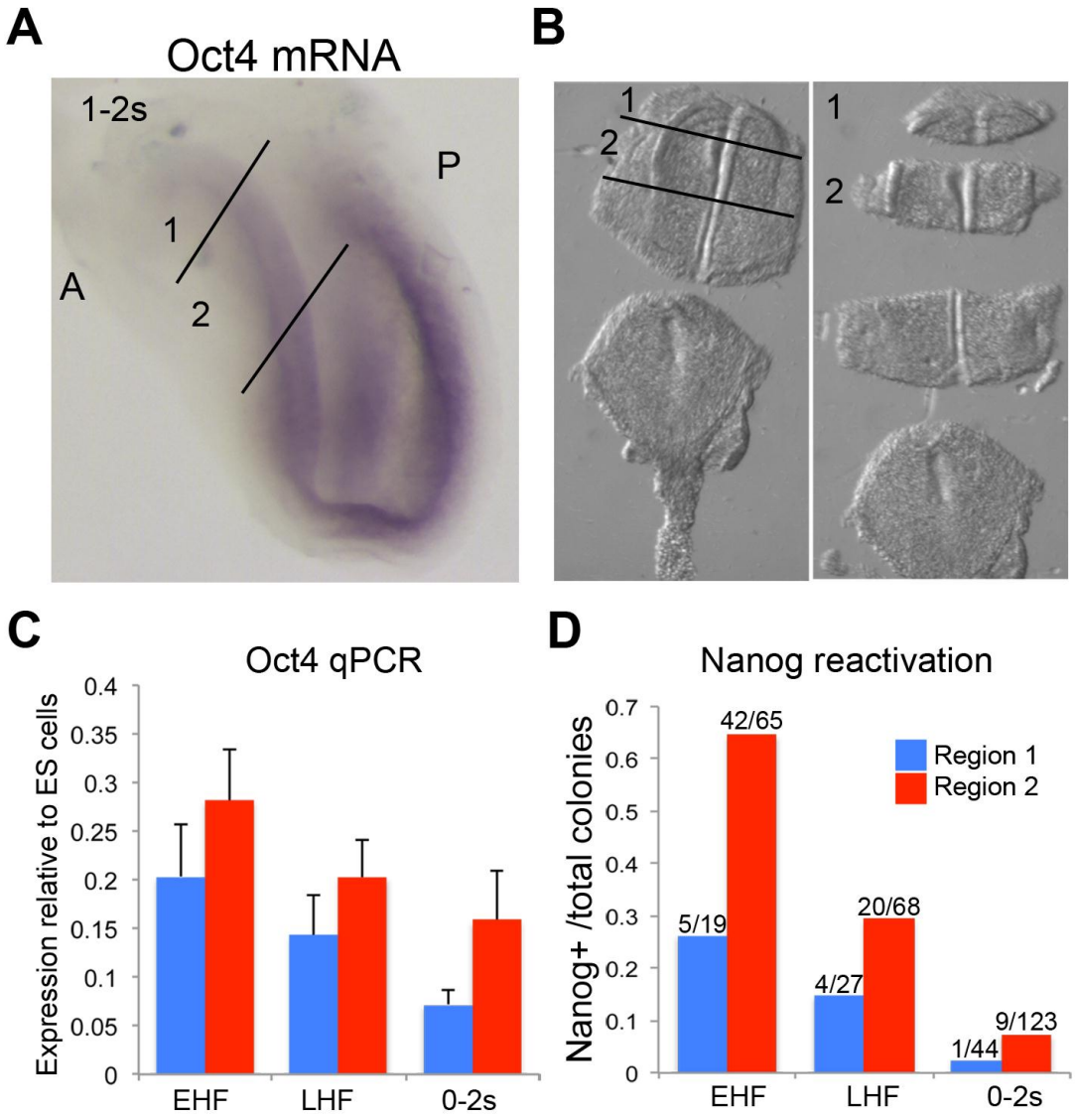


Figure 4. 10 Oct4 levels in the pre-somitogenesis epiblast correlate with efficiency of Nanog reactivation.

(A) Whole-mount in situ hybridization of Oct4 mRNA levels in a 1-2s stage embryo. Regions 1 and 2 used in B and C are indicated by lines. A, anterior; P, posterior.

(B) Micro-dissection of regions 1 and 2 from a 1-2s stage embryo.

(C) Oct4 mRNA expression in pooled samples of regions 1 and 2 micro-dissected from embryos of the indicated stages. Data are mean±s.e.m.

(D) Frequency of Nanog immuno-fluorescent colonies from regions 1 and 2, isolated from pooled embryos of the indicated developmental stages and explanted in DMEM-F12β/KOSR/Activin/bFGF for 48 hours. Number of Nanog+ colonies/total scored is indicated.

In order to test the hypothesis that reduced Oct4 levels results in loss of primed pluripotency, Oct4 levels were manipulated in somitogenesis-stage embryos using mice carrying a doxycycline inducible Oct4 transgene (TgOct4) (Hochedlinger et al., 2005). Anterior (forebrain) and posterior (primitive streak) regions of somitogenesis stage TgOct4/+;rtTA/+ embryos were tested for their ability to reactivate Nanog and generate EpiSC lines in the presence or absence of doxycycline (Figure 4.11). Remarkably, *Nanog*:GFP was induced within 24 hours in explants of E8.5-E10.5 stage embryos cultured in EpiSC conditions exclusively in the presence of doxycycline (Figure 4.11A). Moreover, Sox2 and Nanog proteins were robustly expressed by 48 hours (Figure 4.11B). Interestingly, the frequency of Nanog reactivation declined with the progression of embryonic development (Figure 4.11A). E13.5-E14.5 embryo explants cultured in the presence of doxycycline did not yield Nanog-positive colonies at 48 hours, however, a few Nanog-positive colonies appeared after prolonged culture (120 hours; Figure 4.11A). Consistent with this, E15.5 embryo explants did not yield Nanog-positive colonies even after 120 hours of culture (Figure 4.11A). Therefore, at the onset of somitogenesis Oct4 levels drop below the threshold permissive for primed pluripotency, at this point, Nanog expression and EpiSC forming potential remain sensitive to raised Oct4 levels, but their responsiveness decreases gradually with the progression of embryonic development.

Although the speed and efficiency of Nanog reactivation decreased with the progression of development, induction of the Oct4 transgene in TgOct4/+;rtTA/+ embryos allowed for the derivation of EpiSC lines from E9.0-13.5 (Figure 4.11C-D).

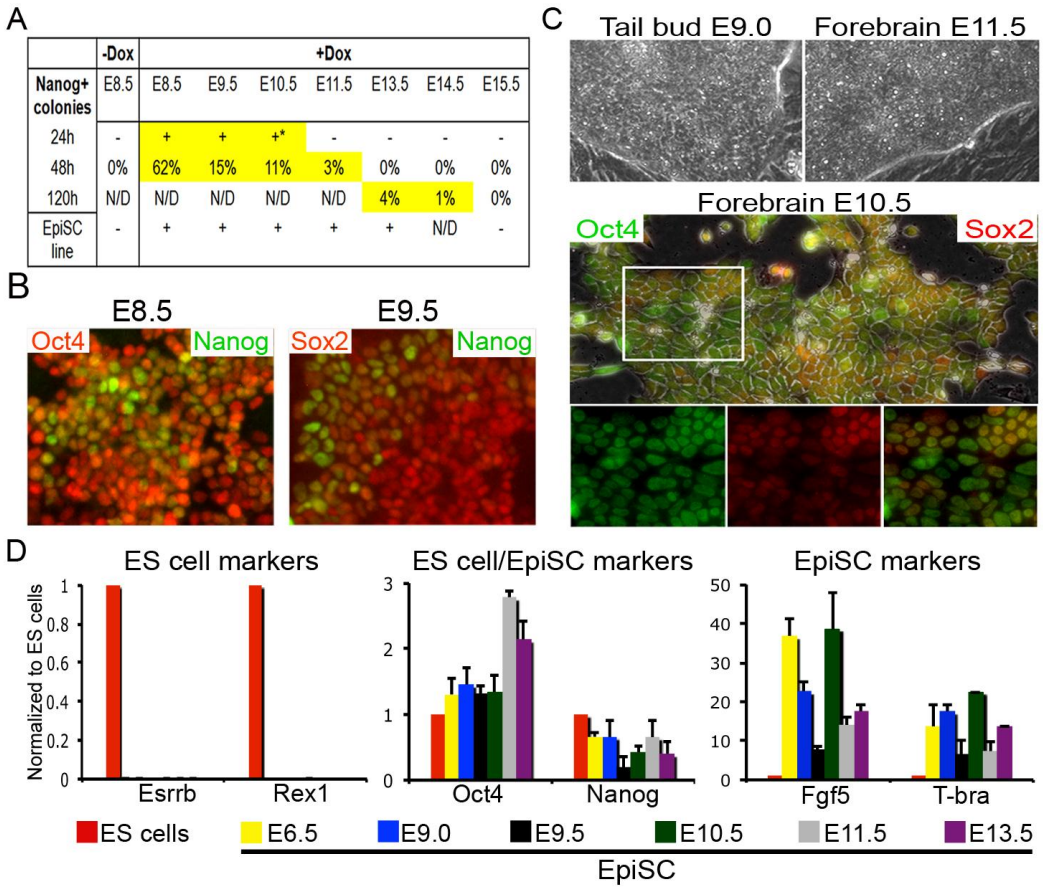


Figure 4. 11 Oct4 mediates Nanog reactivation and restores EpiSC formation ability in somitogenesis stage embryos.

(A) Scoring of Nanog$^+$ colonies from explanted TgOct4/+;rtTA/+ forebrain regions of the indicated stage embryos cultured in the presence or absence of doxycycline. Successful (+) or unsuccessful (–) *Nanog*:GFP reactivation or EpiSC line derivation respectively. Asterisk indicates only one Nanog$^+$ colony was observed after 24h. N/D, not determined. Number of colonies scored: E8.5 +Dox, n□68; E8.5 -Dox, n=53; E9.5, n=648; E10.5, n=675; E11.5, n=143; E13.5, n=220; E14.5-48 hours, n=283; E14.5-120 hours, n=428; E15.5-48 hours, n=145; E15.5-120 hours, n=660.

(B) Nanog and Oct4 or Sox2 immunofluorescence in E8.5 and E9.5 forebrain explants in the presence of doxycycline.

(C) TgOct4/$^+$;rtTA/$^+$ EpiSC lines. Top: Bright field images showing morphology of representative lines. Bottom: Oct4 and Sox2 immunofluorescence. Box indicates the region magnified below.

(D) mRNA expression in established EpiSC lines derived from the indicated embryonic stages. Data are mean±s.e.m of 3 biological replicates.

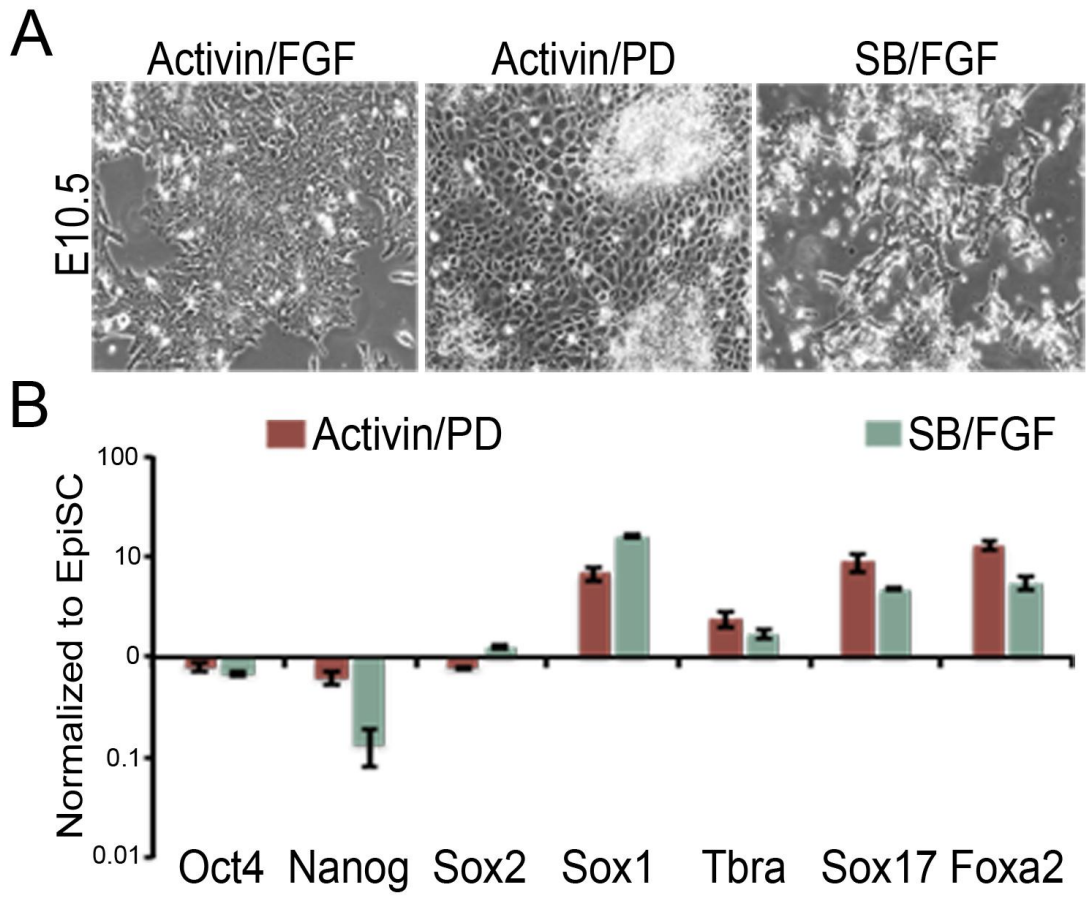


Figure 4. 12 Growth factor dependency in EpiSC derived from somitogenesis stage embryos.

(A) Brightfield images of Oct4-induced E10.5 EpiSC treated for 7 days with a selective inhibitor of Transforming Growth Factor-β (TGF-β) superfamily type I Activin receptor-like kinase (ALK) receptors (SB431542) or a MEK inhibitor (PD0325901).

(B) Gene expression analysis of E10.5 EpiSC after 48 hour treatment with SB431542 or PD0325901. qPCR analyses were determined relative to TATA box-binding protein (TBP) and normalized to untreated E10.5 EpiSC. All qPCR results are the average of at least 3 independent experiments. Error bars: S.E.M.

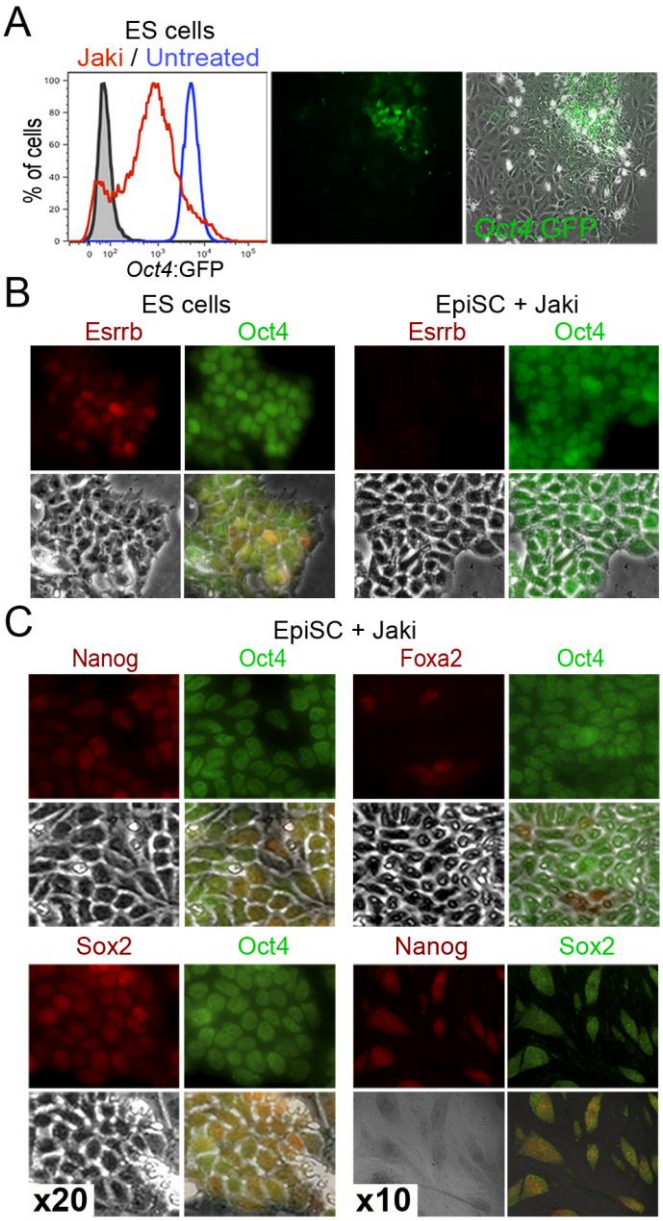


Figure 4. 13 JAK inhibition in EpiSC is permissive for the expression of pluripotency factors.

(A) Oct4GiP ES cells treated with JAK inhibitor I (Calbiochem 420099; 0.6mM) for 5 days in GMEMβ/FCS/LIF downregulate Oct4:GFP. FACS analysis (left panel), *Oct4*:GFP fluorescence (middle panel) and fluorescence optics (right panel).

(B) Immunohistochemistry for Esrrb in Oct4-induced E10.5 EpiSC and control ES cells.

(C) Immunohistochemistry for Nanog, Oct4, Sox2 and Foxa2 in Oct4-induced E10.5 EpiSC after their cultured in N2B27/Activin/FGF/JAKi for 1 month. Cells display expression of Nanog, Oct4, Foxa2 and Sox2 proteins (20x); robust expression of Nanog and Sox2 proteins in Oct4-induced E10.5 EpiSC colonies (10x).

E9.0-13.5 EpiSC displayed typical undifferentiated morphology, while presenting robust expression of Oct4 and Sox2 proteins (Figure 4.11C). Furthermore, E9.0-13.5 EpiSC lines showed marker gene expression similar to pre-somitogenesis stage EpiSC (Figure 4.11D). Importantly, the self-renewal of EpiSC derived from somitogenesis stage embryos was dependent on Activin and FGF signaling (Figure 4.12). Treatment with either an Activin (SB431542) or MEK (PD0325901) resulted in the differentiation of E10.5 EpiSC (Figure 4.12A). qPCR analysis showed that lineage specific markers were upregulated in E10.5 EpiSC treated with either an Activin (SB431542) or MEK inhibitor (PD0325901) (Figure 4.12B). Notably, inhibition of JAK/STAT3 signaling did not affect the prolonged culture (~month) of EpiSC derived from somitogenesis stage embryos, providing further evidence that these are *bona fide* EpiSC (Figure 4.13). Moreover, teratocarcinoma-forming capacity was retained after prolonged culture in the presence of a JAK inhibitor (Figure 4.14). To test whether Oct4 induces teratocarcinoma-forming potential without prior derivation of EpiSCs, freshly dissected TgOct4/+;rtTA/+ forebrain regions from 5-11s stage embryos were grafted to the kidney capsule of wild-type hosts. These animals were kept in the presence or absence of doxycycline in the drinking water for the duration of the assay (Figure 4.14A-B). After a month, two out of three doxycycline-treated grafts had formed large teratocarcinomas containing derivatives of all three germ layers (Figure 4.14B-C). Conversely, untreated tissue formed only small, differentiated tumours (Figure 4.14B). In order to establish the presence of self-renewing cells in the E10.5 EpiSC derived-tumours, secondary EpiSC were isolated (Figure 4.15). After 4 passages, these lines showed characteristic EpiSC morphology and expressed Oct4, Nanog and Sox2 (Figure 4.15B-C). Thus, proving that E10.5 EpiSC derived-tumours contained self-renewing cells which

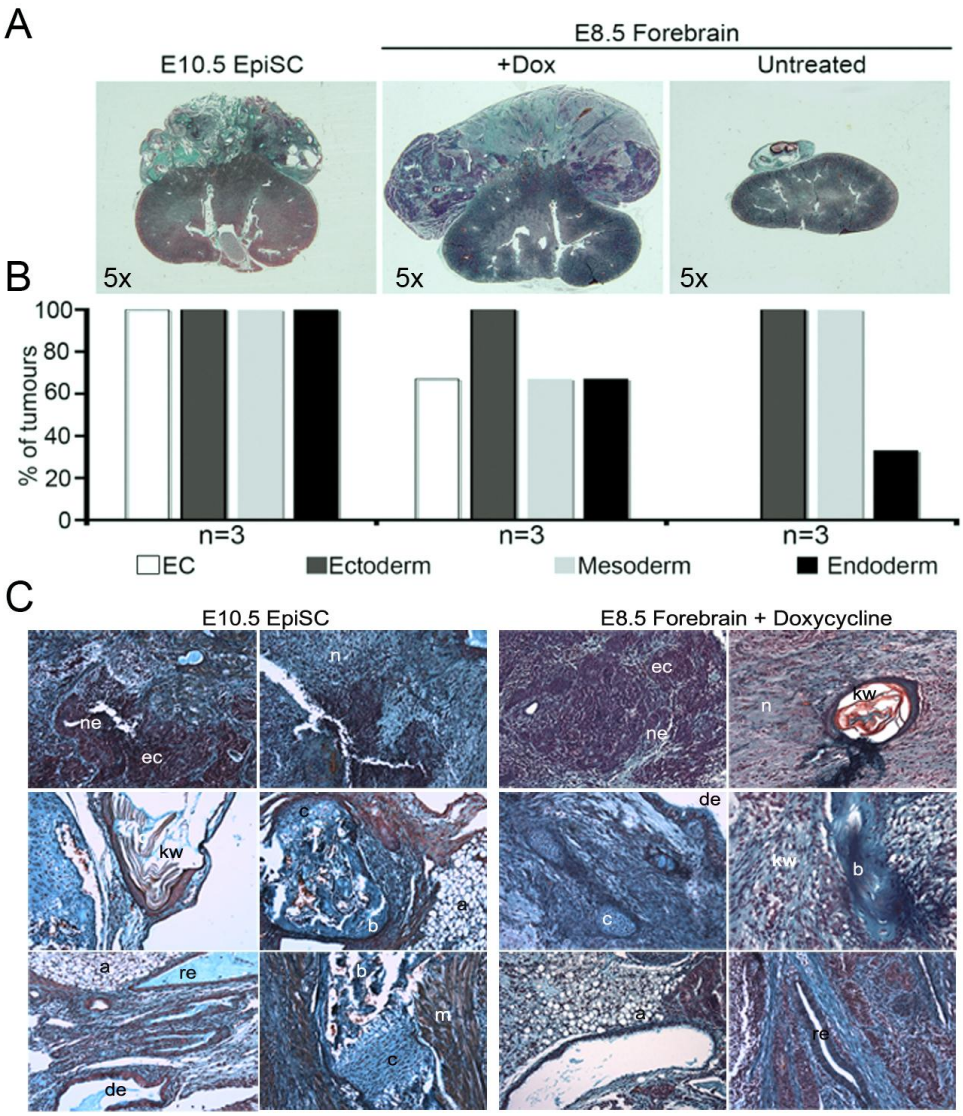


Figure 4. 14 Enforced Oct4 restores pluripotency in somitogenesis-stage embryos.

(A) Teratocarcinoma formation assay using E10.5 EpiSC and freshly dissected forebrain (E8.5). E10.5 EpiSC, cultured for a month in JAK inhibitor I, was injected under the kidney capsule of recipient mice. Freshly dissected E8.5 forebrain was transplanted under the kidney capsule of doxycycline-treated or untreated mice.

(B) Scoring of germ layer differentiation of the indicated EpiSC or embryonic tissue following a teratocarcinoma-forming assay.

(C) Representative examples of tissue types contained within teratocarcinomas obtained either after engraftment of E10.5 EpiSCs (left) or E8.5 rtTA/$^{+}$:Oct4/$^{+}$ forebrain (right) under the kidney capsule of Doxycycline-treated mice. ne, immature neuroepithilium; ec, embryonal carcinoma; n, mature nervous tissue; kw, keratin whorl; b, bone; c, chondrocytes; a, adipose tissue; de, digestive epithelium; re, respiratory epithelium; m, muscle.

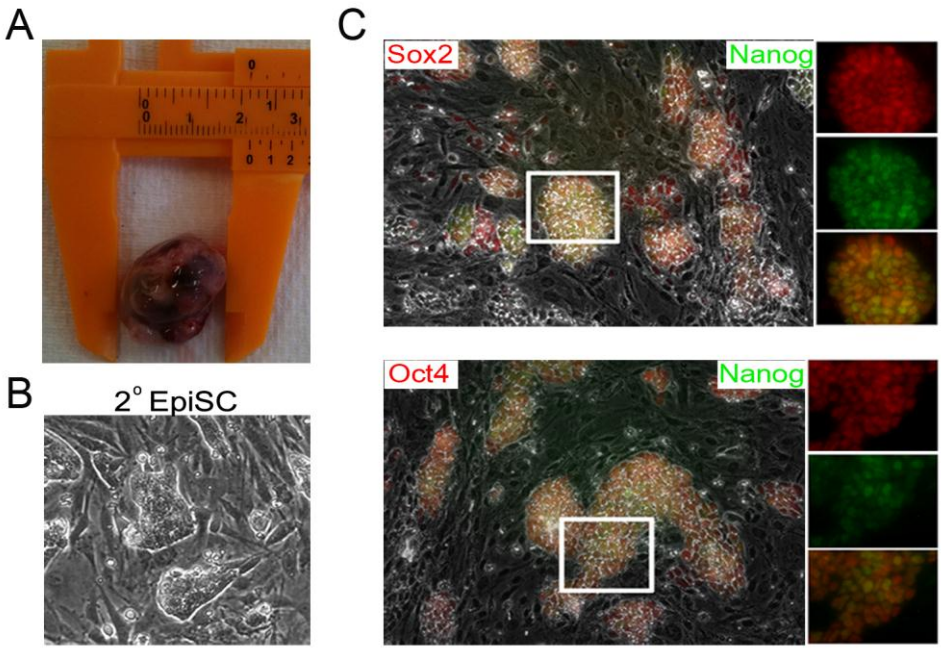


Figure 4. 15 EpiSC readily generate teratocarcinomas and 2° EpiSC.

(A) Freshly dissected kidney with Oct4-induced E10.5 EpiSC derived tissue.

(B) Brightfield image of secondary EpiSCs isolated from E10.5 EpiSC teratocarcinoma.

(C) Immunofluorescence for Oct4/Nanog or Sox2/Nanog in secondary EpiSC. Secondary EpiSC were cultured for 4 passages in DMEM-F12β/KOSR/Activin/bFGF in the absence of doxycycline. Boxed areas are magnified in the right hand panels.

can be propagated *in vitro*. Together these results indicate that a threshold level of Oct4 is necessary to sustain primed pluripotency, and suggests that in somitogenesis stage embryos, restoration of these levels allows the reactivation of the pluripotency network in non-pluripotent cells.

4.6 Changes in nucleosome occupancy accompany the loss and reacquisition of pluripotency

The mechanisms involved in the loss and reacquisition of primed pluripotency were investigated. Since chromatin changes participate in gene regulation, nucleosome positioning at the regulatory regions of *Oct4*, *Nanog* and *Sox2* was examined (Figure 4.16). Recently it has been shown that active promoters/transcription start sites are preferentially isolated using FAIRE (Giresi et al., 2007; Nagy et al., 2003). FAIRE was used to analyse nucleosome-depletion in the regulatory regions of the core pluripotency genes (Giresi et al., 2007). FAIRE analyses showed prominent changes between pre-somite and early somite stage embryos. Interestingly, a reproducibly significant reduction was found in the overall accessibility of chromatin at both *Oct4* and *Nanog* regulatory regions in whole embryos (Figure 4.16A), which correlated with the observed changes in transcript levels (see section 4.1). Chromatin closure was reversed in 2-8s stage tissue following Oct4 induction and explantation in EpiSC conditions after 24 hours (Figure 4.16B). Notably, Oct4 regulatory elements did not show as extensive reopening as *Nanog* (Figure 4.16B), suggesting differences in the regulation of pluripotency network components. Moreover, EpiSC culture conditions alone can

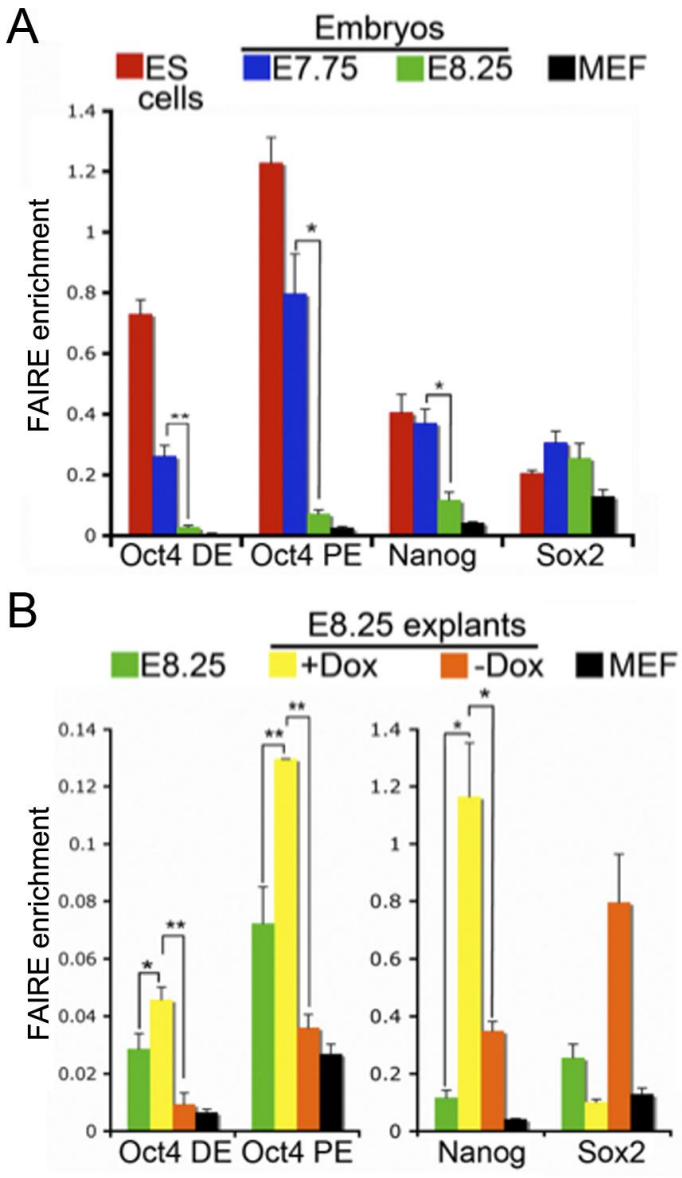


Figure 4. 16 Chromatin changes at pluripotency transcription factor genes.

(A) FAIRE enrichment (ratio of test:reference DNA, normalized to β-actin) of pluripotency transcription factor genes in presomite (LB-EHF; E7.75) and somitogenesis-stage (two to eight somites; E8.25) embryos compared with ES cells and MEFs. Nanog and Sox2 promoters and the distal enhancer (DE) and proximal enhancer (PE) of Oct4 were analysed.

(B) FAIRE of E8.25 embryo explants cultured for 24 hours in EpiSC conditions in the presence (+Dox) or absence (–Dox) of doxycycline compared with E8.25 tissue or MEFs. Data are mean±s.e.m. Significant differences in A and B were calculated using a t-test: *P>0.05; **P>0.01.

provoke chromatin reopening at *Nanog* in the absence of ectopic Oct4 expression and Nanog reactivation (Figure 4.16B). This suggests that growth factors present in EpiSC culture conditions can stimulate re-opening of the *Nanog* promoter, however, binding of Oct4 to the Oct/Sox motif is required to stimulate Nanog transcription.

4.7 Progressive decline in pluripotency reactivation correlates with increased methylation at Nanog

The changes in the accessibility of *Oct4* and *Nanog* do not fully account for the decreasing efficiency to reactivate Nanog in presence of elevated Oct4 expression. The previous observation suggests that additional mechanisms may be involved in the stable shutdown of pluripotency network. Therefore, the extent of CpG methylation at the *Nanog* promoter was examined in E7.5-E14.5 embryos (Figure 4.17). It has previously been shown that the *Nanog* promoter exhibits higher levels of methylation in MEFs compared with ES cells (Imamura et al., 2006). Interestingly, it was found that methylation at the *Nanog* promoter were low at both presomite and early somite stages, however, methylation increased as development progressed (Figure 4.17). Moreover, analysis of the Oct4-induced E13.5 EpiSC line exhibited methylation levels comparable to pre-somite E7.5 embryos (Figure 4.17; Bisulfite sequence analysis of the *Nanog* promoter were performed by Dr. Anestis Tsakiridis). These findings indicate that Oct4 mediated reactivation of *Nanog* and acquisition of primed pluripotency is accompanied by a decrease in methylation at *Nanog*. Moreover, increase methylation at *Nanog* correlates with the progressive decrease in efficiency to reactivate this key pluripotency gene and require primed pluripotency.

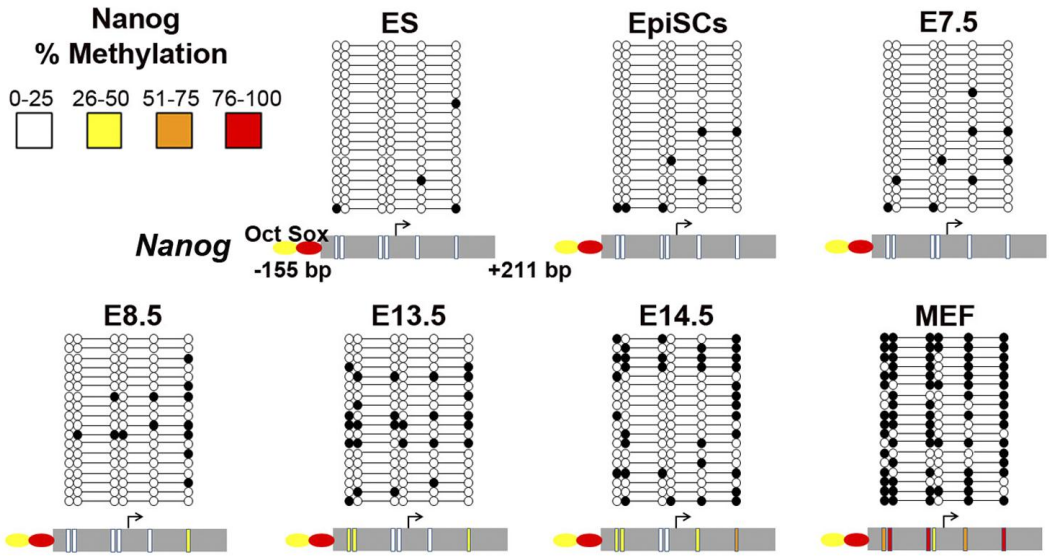


Figure 4. 17 Methylation analysis of Nanog during embryonic development.

Methylation analysis of the Nanog proximal region (grey rectangle) by bisulphite sequencing of DNA extracted from whole embryos at the indicated stages. Methylation levels in ES cells, MEFs and an established E13.5 forebrain EpiSC line are shown for comparison. Filled (methylated) or empty (unmethylated) circles: CpGs on individual DNA molecules (horizontal lines). Below each stage, vertical bars superimposed on the Nanog promoter represent the proportion of methylated CpGs at each position. Green/purple ovals indicate Oct/Sox-binding sites. Arrows indicate the transcriptional start site (TSS). Bisulfite sequence analysis of the Nanog promoter was performed by Dr. Anestis Tsakiridis.

4.8 Discussion

Previous studies have shown that somatic pluripotency, as indicated by the ability to generate teratomas, is lost in the mouse embryo around E8.5 (Beddington, 1983; Damjanov et al., 1971). However, the precise timing and mechanism involved in this process have not been defined. Oct4, Sox2 and Nanog are expressed in the pluripotent post-implantation epiblast and in derivative EpiSCs (Brons et al., 2007; Tesar et al., 2007). However, little is known about the role of these core pluripotency regulators in primed pluripotency. The work presented in this chapter provides new insights into the mechanisms involved in the maintenance and loss of post-implantation pluripotency.

4.8.1 Pluripotency during post-implantation development

Following implantation, the expression of Nanog and Oct4 gradually decrease until becoming undetectable at 3-5 somite embryo and 12-15 somite embryo, respectively. Conversely, Sox2 expression increases during gastrulation and eventually becomes restricted to the neurectoderm. Prior to somitogenesis expression of the core pluripotency factors, Oct4, Sox2, and Nanog, is regionalized. It is puzzling that in the E7.5 embryo, where pluripotency is a general property of the epiblast, these genes are not ubiquitously expressed. Indeed, three expression domains can be identified at E7.5, a Sox2-Oct4 domain at the distal-anterior part of the epiblast, a Nanog-Oct4 domain in the posterior-proximal region, and a region in which all of these three markers overlap, forming a diagonal band from anterior-proximal to posterior-distal. It is noteworthy that the Sox2-Oct4 domain probably

corresponds to neurally fated cells, therefore, it is not surprising that Nanog is down-regulated in this region as it is well documented that it blocks neural differentiation (Ying et al., 2003b). On the other hand, Nanog-Oct4 co-occupy a domain that seems to mark non-neural fate. Nonetheless, these regions are all pluripotent when tested by teratocarcinoma assays or EpiSC derivation. This suggests that at E7.5 pluripotency is latent, but not an overt property of epiblast cells.

Nodal expression in the primitive streak disappears just before the stable inactivation of Nanog. It is therefore possible that Nodal could be responsible for the restricted expression of Nanog in the embryo, and its loss could contribute to the disappearance of Nanog expression. In support of this, *Nanog*:GFP expression in cells explanted in EpiSC conditions is dependent on stimulation of the Activin signaling pathway and moderately affected by the FGF pathway. Moreover, the null mutants for Nodal show a downregulation in Nanog expression (Mesnard et al., 2006). Therefore, Nanog expression in the post-implantation epiblast is likely to depend on Nodal signaling.

Given the fact that most cells are fate-restricted at E7.5 and that in the adult pluripotent cells are neoplastic, it is intriguing that the embryo preserves pluripotency long after it is required. In principle there are three reasons as to why this occurs: (1) the embryo requires pluripotency until LHF stage, (2) pluripotency must be switched off before the onset of somitogenesis, (3) the downregulation of pluripotency gene expression tracks a major shift in cell states and is merely a convenient timepoint to downregulate all pluripotency genes. The extinction of

pluripotency in the epiblast occurs a full day after lineage restriction (Lawson et al., 1991; Tzouanacou et al., 2009). This suggests that pluripotency is not required for development up until somitogenesis, but rather that it is necessary to extinguish pluripotency by this time. Since FGF and activin-like signaling control region-specific patterning during somitogenesis (Kawasumi et al., 2011; Mariani et al., 2008; Naiche et al., 2011; Suzuki-Hirano et al., 2005), the primary rationale for extinction of pluripotency may be to allow orderly progression of organogenesis with a secondary effect being to prevent teratocarcinogenesis. An obvious experiment would be to overexpress a core pluripotency factor like Nanog or Oct4 to test whether the maintenance of pluripotency has any negative effects for the progression of development.

4.8.2 Nanog is not essential for EpiSC derivation and maintenance

At E7.5, cells in the distal-anterior epiblast that do not express Nanog, remain pluripotent when tested for their ability to make EpiSC and teratocarcinomas. However, these experiments are not conclusive, since it is not possible to exclude the possibility that *Nanog* is reactivated in these cells. Therefore, to consolidate this observation, pluripotent cells genetically depleted for Nanog were used. Nanog-null ES cells were differentiated *in vitro* into EpiSC. This categorically showed that Nanog is not essential for the specification and maintenance of primed pluripotency. Furthermore, Nanog levels are higher in ES cells than in EpiSC (Han et al., 2010; Osorno and Chambers, 2011). It is possible that it serves as a peripheral reporter on activation of the pluripotency network. It may

be true that subtle changes in cells are not detected by these assays and more quantitative methods may uncover roles for Nanog. One possibility is that in all systems, Nanog promotes faster or more efficient self-renewal. It was demonstrated that Nanog-null cells have impaired self-renewal when compared to wild type cells (Chambers et al., 2007). However, this does not explain the complete disappearance of the inner cell mass from Nanog-null diapause embryos (Silva et al., 2009). Nanog has been proposed to be necessary to prevent neural differentiation in EpiSC (Vallier et al., 2009). Consistent with this hypothesis is the observation that Nanog marks the region of the epiblast fated for non-neural tissues. Despite not being required for the maintenance of post-implantation pluripotency, Nanog remains a reliable indicator of pluripotency

4.8.3 Ectopic Oct4 resuscitates somatic pluripotency

The correlation between loss of pluripotency and Oct4 downregulation, prompted us to test the effects of Oct4 re-expression in somitogenesis-stage embryos. This has led to the observation of a novel transition state in which cells are no longer pluripotent but can rapidly, and efficiently, reacquire pluripotency upon re-expression of Oct4. The speed and efficiency of the process is further proof of such transition state, since Oct4-mediated reactivation of the pluripotency is far more efficient and faster than somatic cell reprogramming. This transition state is characterised by a closed chromatin structure at key network regulatory elements, and low levels of DNA methylation at *Nanog*. Methylation progressively increases during later development, suggesting that there are two mechanisms to ensure that

pluripotent cells are eliminated from the somitogenesis-stage embryo. The first is a rapid chromatin-based shutdown that is susceptible to perturbation by variation in the levels of pluripotency network components, and the second, a slower, more permanent, DNA methylation-based stabilization of the non-pluripotent state. Importantly, a previous study has shown that ectopic expression of Oct4 in 3-week old mice does not result in teratocarcinoma formation, suggesting that pluripotency was not reactivated (Hochedlinger et al., 2005).

Chapter 5

Transcription factor regulation of epiblast pluripotency

5.1 Introduction

The mouse epiblast has been shown to present at least two distinct pluripotent states (Brons et al., 2007; Evans and Kaufman, 1981; Martin, 1981; Tesar et al., 2007). ES cells and EpiSC express the core transcription factors Oct4, Sox2 and Nanog (Brons et al., 2007; Tesar et al., 2007). However, these populations differ in several aspects, such as morphology, clonal propagation abilities, requirements for exogenous factors to promote self-renewal and their ability to re-colonize the embryo. Global gene expression analysis suggests that the ES cells and EpiSCs display differences in their transcription factor circuitry (Brons et al., 2007; Tesar et al., 2007).

5.2 ES cells and EpiSC are distinct pluripotent states

Nanog expression first appears *in vivo* around the morula stage, thereafter Nanog is strongly expressed in the nascent epiblast of the ICM (Chambers et al., 2003; Mitsui et al., 2003). *In vivo* analysis of Nanog-null embryos indicates that Nanog is essential for the specification of the pluripotent epiblast (Silva et al., 2009). Once the epiblast has been specified, Nanog mRNA is downregulated around the

peri-implantation stage (Figure 5.1). This is also recapitulated using a *Nanog*:GFP reporter mouse line (Figure 5.1). Following implantation, Nanog mRNA is subsequently detected in the epiblast (Figure 5.1, see also chapter 4). These observations are in contrast to the continued expression of Oct4 and Sox2 during implantation and suggest that Nanog may have a brake function that must be removed in order for the changes in pluripotent phenotype that occur at implantation to proceed.

ES cells can be differentiated *in vitro* into EpiSC by changing the growth factors in the medium from LIF/FCS to Activin/FGF (Figure 5.2; (Guo et al., 2009). ES cell transcription factors such as Klf2, Klf4, Esrrb, Nr5a2, Tbx3, Rex1 and Nanog are downregulated during this transition (Figure 5.2B). Moreover, the rates of change in expression of these factors differ. For instance, Klf4 expression plummets by day 1. Esrrb expression is reduced to around 30% of its expression by day 1, subsequent expression remained low. Klf2 and Rex1 display different dynamics, the expression of these genes is halved around days 2-3 and further decreasing by days 3-4 to around 25% of their expression in ES cells. Nanog and Tbx3 are progressively downregulated in the conversion of ES cells into EpiSCs. The expression of Klf2, Klf4, Esrrb, Nr5a2 and Rex1 is undetectable in established EpiSC. Sox2, Tbx3 and Nanog are expressed at lower levels in EpiSC when compared to ES cells. The levels of Oct4 show small changes when ES cells are forced to differentiate into EpiSC (Figure 5.2; (Han et al., 2010)).

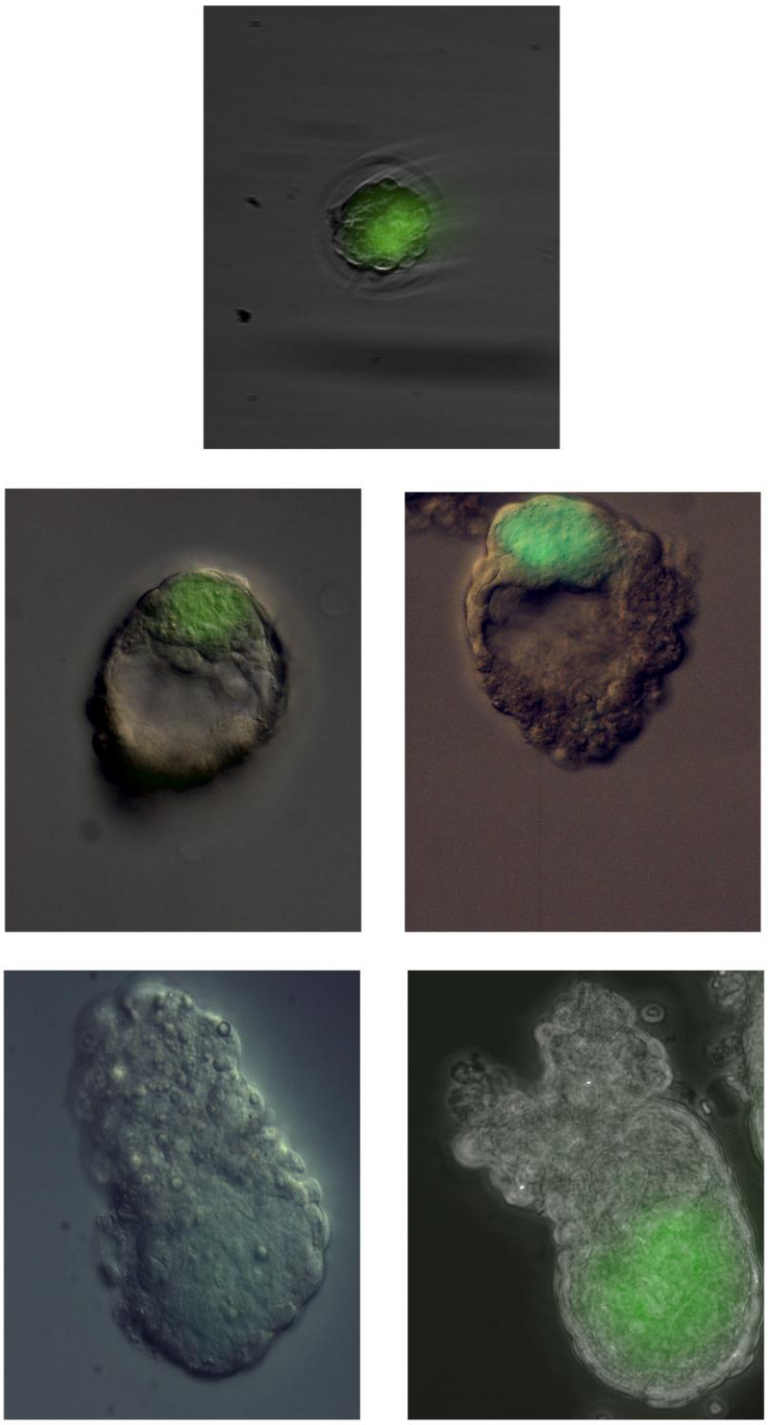


Figure 5. 1 Nanog expression in peri-implantation embryos.

Nanog:GFP expression during the peri-implantation period. Top panel, Nanog:GFP expression in compacted morula. Middle panel, Nanog:GFP expression in early (left) and hatched blastocyst (right). Bottom panel, Nanog:GFP expression in E4.5 (left) and E5.0 (right) embryos.

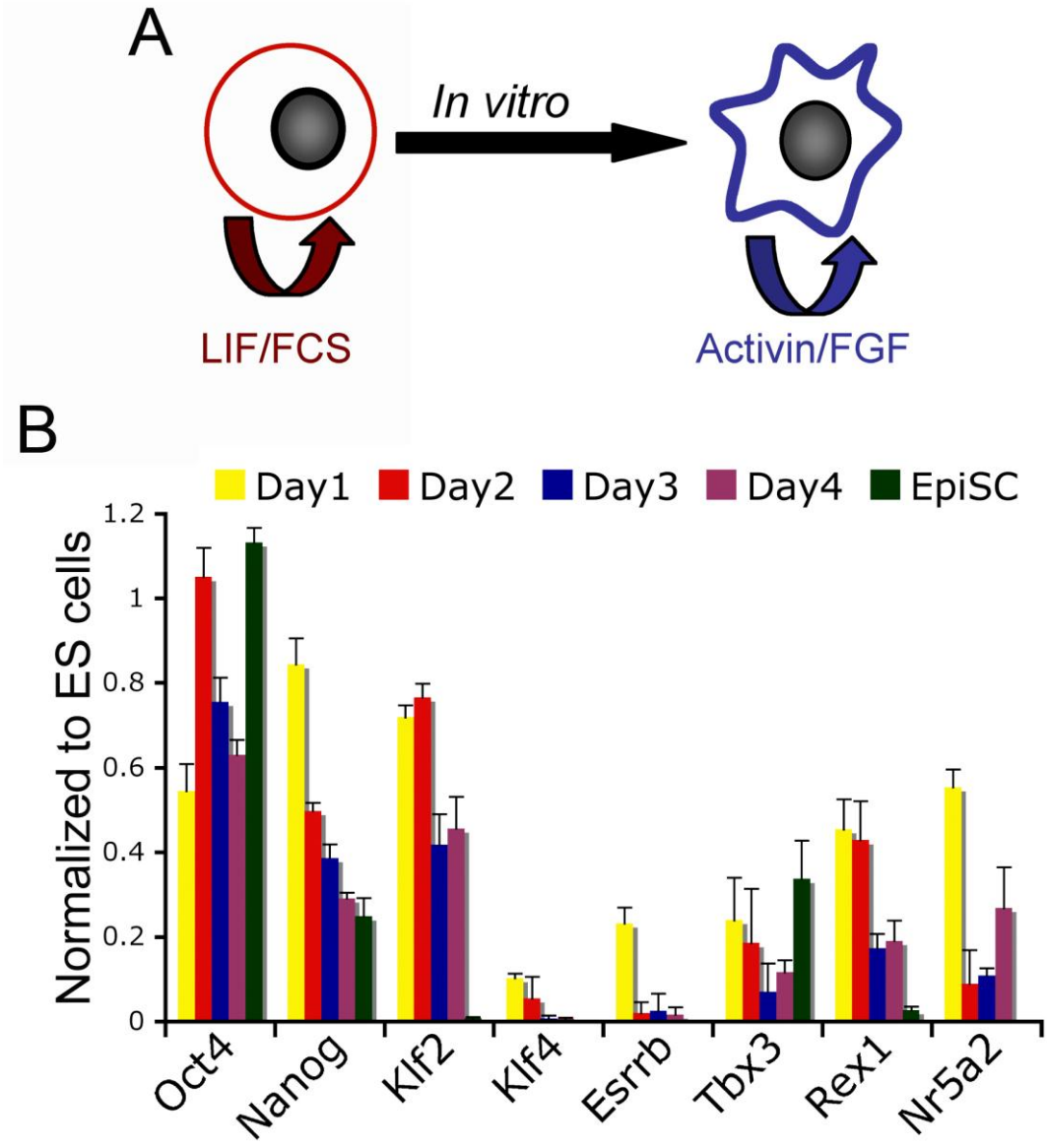


Figure 5. 2 Transition from naive to primed pluripotency.

(A) The developmental changes that occur between the nascent epiblast found in the blastocyst and the post-implantation epiblast can be recapitulated *in vitro* by changing growth factor supplements to the medium.

(B) Gene expression changes occurring upon media switch to N2B27/Activin/Fgf. qPCR showing the changes that occur to transcription factor mRNAs when ES cells differentiate into EpiSC. ES cells were plated in GMEMβ/FCS/LIF. 24 hours later the media was switched to N2B27/Activin/bFGF. qPCR analyses were determined relative to TBP and normalized to the ES cell level of 1.0. All qPCR results are the average of at least 3 independent experiments. Error bars: S.E.M.

Flow cytometry analysis of Oct4GiP ES cells (Yeom et al., 1996) demonstrated that expression of this pluripotency regulator does not vary significantly during the transition from ES cells to EpiSC (Figure 5.3A). TNG ES cells (Chambers et al., 2007) were also analysed using the same experimental strategy. *Nanog*:GFP expression was found to gradually decrease as ES cells differentiated into EpiSC (Figure 5.4). An even more rapid shift was observed using an Esrrb-TdTomato reporter line (obtained from Nicola Festuccia). Esrrb-TdTomato expression is completely shut off after 4 days of culture in Activin/Fgf (Figure 5.5).

Nanog overexpression hampers the ability of ES cells to differentiate into the neural lineage (Chambers et al., 2003; Ying et al., 2003a). Together with the observation that Nanog expression is lower in EpiSC (Figure 5.2; Figure 5.4; (Han et al., 2010)), this prompted the hypothesis that Nanog downregulation is critical for the transition into EpiSC. To achieve this, ES cells that overexpress different levels of Nanog were tested for their ability to make EpiSCs (5x Nanog, EF4; 2x Nanog, RCN; (Chambers et al., 2003; Chambers et al., 2007; Yates and Chambers, 2005). A negative correlation between the Nanog levels and the ability of ES cells to commit to an EpiSC state was observed (Figure 5.6). Cells that express 5x the level of Nanog fail to display EpiSC morphology after prolonged culture in EpiSC medium (Figure 5.6A). Two independent ES cell lines that overexpress Nanog were cultured in EpiSC media for eight passages and failed to downregulate ES cell-specific pluripotency factors, Rex1, Klf4 and Esrrb (Figure 5.6B). Moreover, in contrast to controls, ES cell lines overexpressing Nanog resist upregulation of differentiation markers Foxa2, T brachyury and Fgf5 (Figure 5.6B).

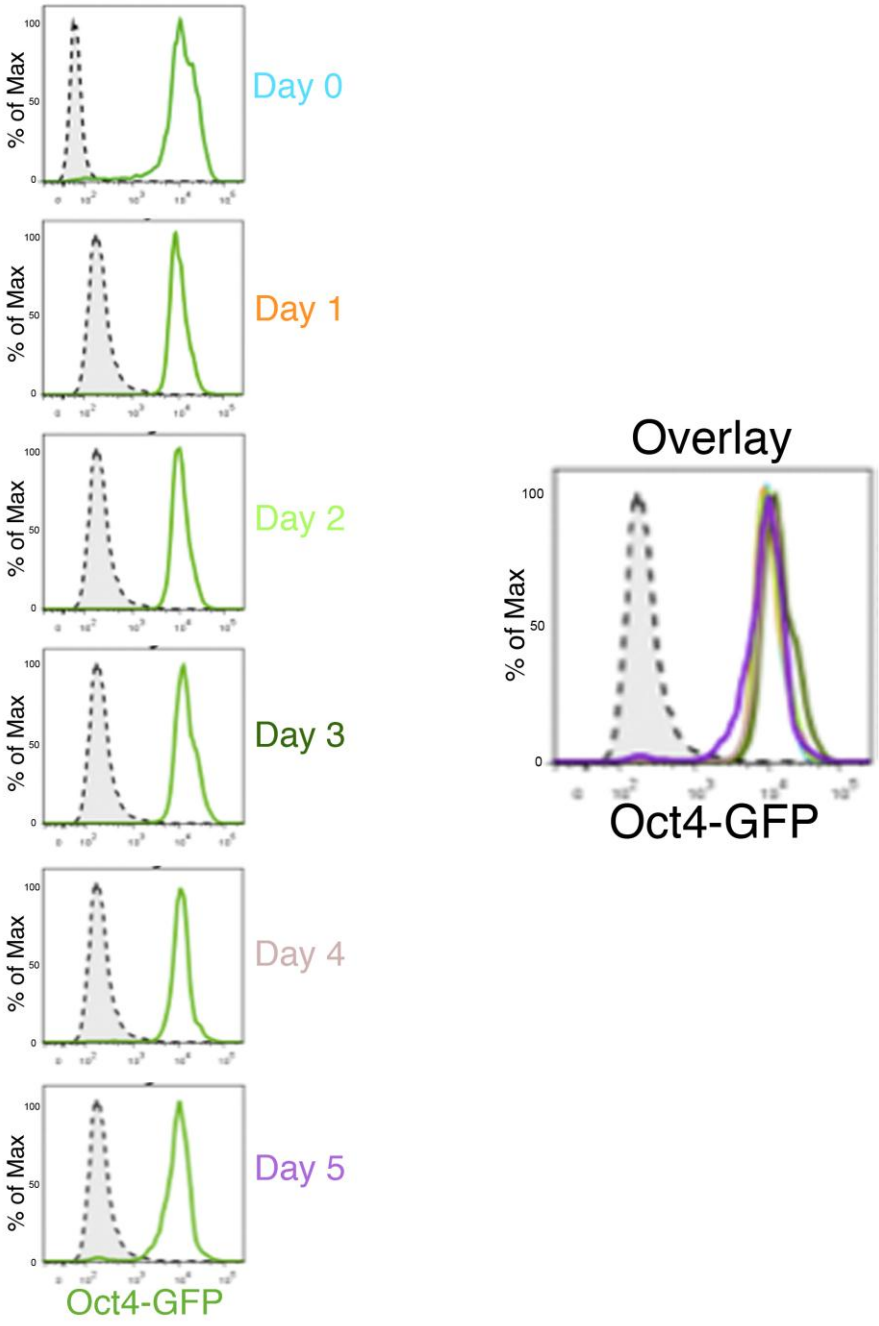


Figure 5. 3 *Oct4*:GFP expression during the differentiation of ES cells into EpiSC.

Flow cytometry analysis of Oct4GiP cells during the differentiation of ES cells to EpiSC. Oct4GiP cells were plated on GMEMβ/FCS/LIF, the following day the medium was switched to N2B27/Activin/bFGF and harvested every 24 hours for analysis by flow cytometry.

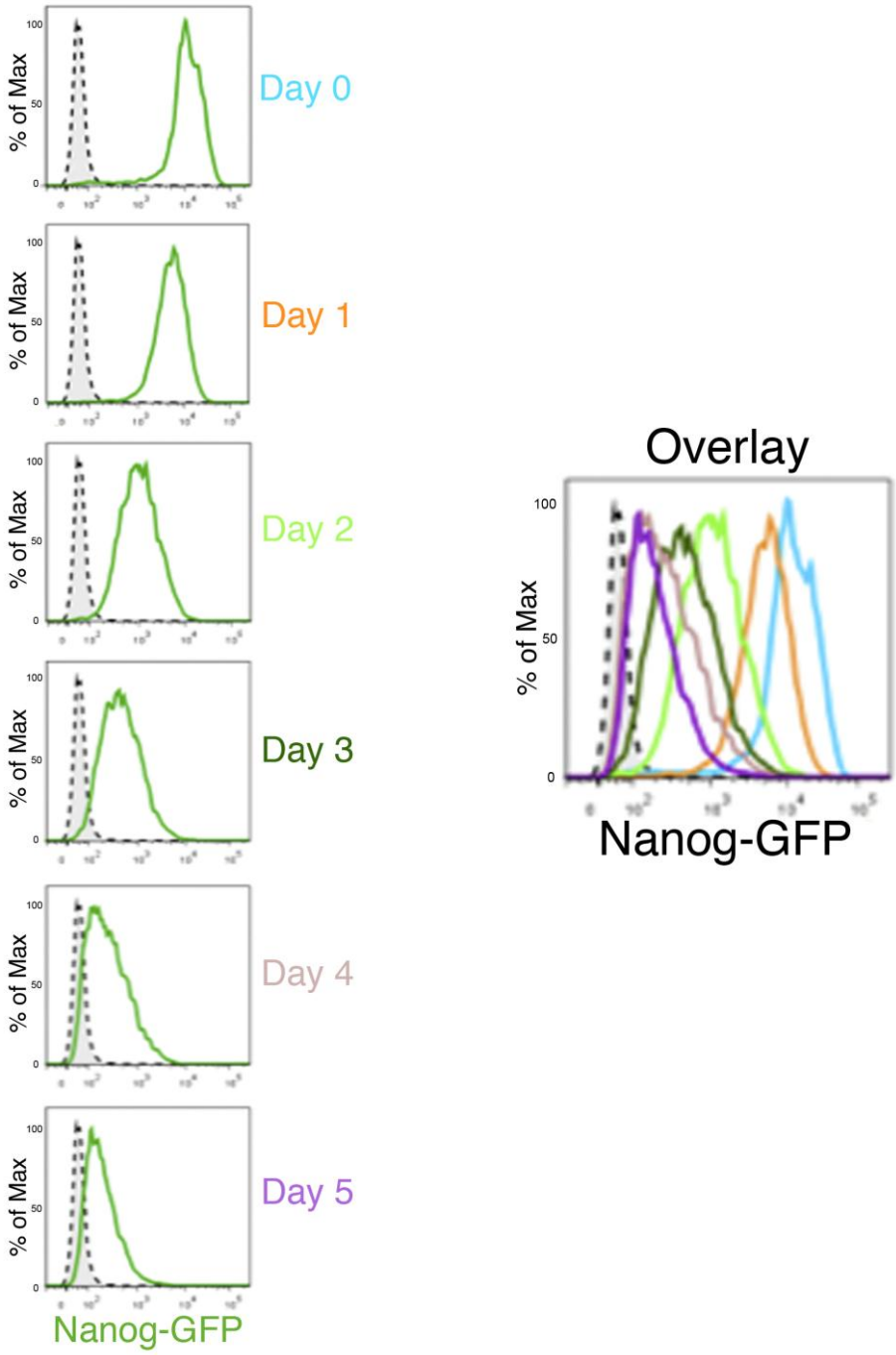


Figure 5. 4 *Nanog*:GFP expression during the transition of ES cells into EpiSC.

Flow cytometry analysis of *Nanog*:GFP cells during the differentiation of ES cells to EpiSC. TNG cells were seeded in GMEMβ/FCS/LIF, the following day the medium was switched to N2B27Activin/ bFGF and harvested every 24 hours for analysis by flow cytometry.

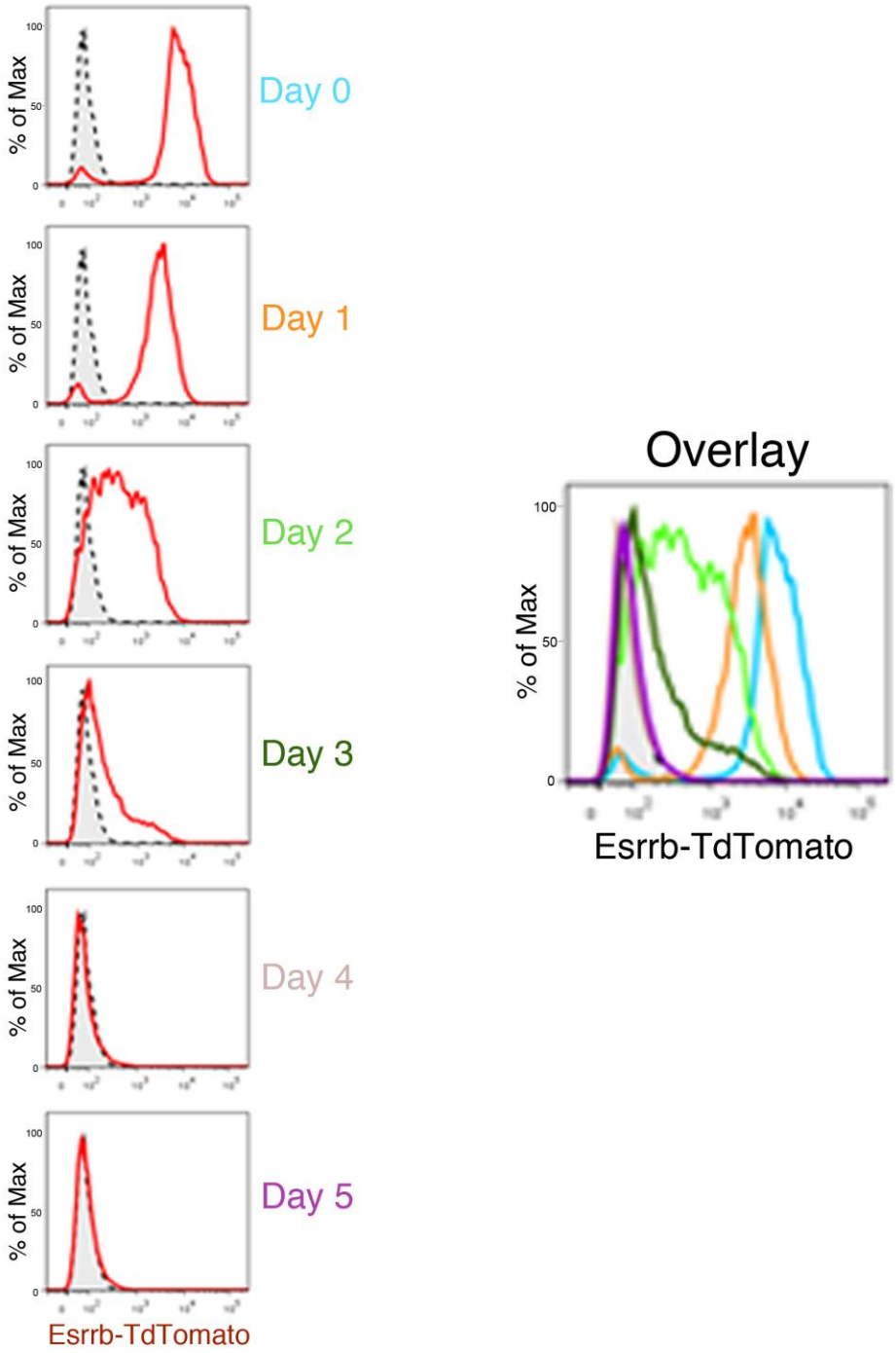


Figure 5. 5 *Esrrb*:TdTomato expression during the differentiation of ES cells into EpiSC.

Flow cytometry analysis on *Esrrb*:Tdt ES cells during differentiation of ES cells to EpiSC. Esrrb-TdTomato cells were seeded in GMEMβ/FCS/LIF, the following day the media was switched to N2B27/Activin/bFGF and harvested every 24 hours for analysis by flow cytometry. *Esrrb*:Tdt ES cells were generated by Nicola Festuccia.

Since EpiSCs differentiate when exposed to ES cell media (Guo et al., 2009), this can be used to assay the effectiveness of differentiation of cells to an EpiSC state. Unlike controls, Nanog-overexpressing cells formed ES cell colonies when returned to ES cell media, even after prolonged cultured in EpiSC conditions (Figure 5.6C). This indicates that forced expression of Nanog in ES cells can block their full transition into an EpiSC state.

Similar to Nanog, Esrrb overexpression can confer LIF-independent self-renewal (Festuccia et al., 2012; Zhang et al., 2008). Therefore the ability of elevated levels of Esrrb to prevent the differentiation of EpiSC from ES cells was tested. A clonal line of Esrrb overexpressing ES cells (EfEsrrbc1), shown to be capable of cytokine independent self-renewal (Festuccia et al., 2012), was tested for their ability to commit to an EpiSC state. However, in contrast to Nanog, sustained expression of Esrrb in ES cells did not block differentiation into EpiSC. Two clones of E14Tg2a overexpressing Esrrb (EfEsrrbc1 and EfEsrrbc2; ES cells generated by Dr. Adam Yates) were cultured in media supplemented with Activin/Fgf for more than 3 passages. Without exception all the clones tested displayed EpiSC morphology (Figure 5.7A). Furthermore, gene expression analysis indicated that these cells upregulated expression of lineage specific markers, while switching off ES cell specific markers (Figure 5.7B). Ectopic expression of Esrrb in ES cells is insufficient to prevent the differentiation induced by Activin/Fgf.

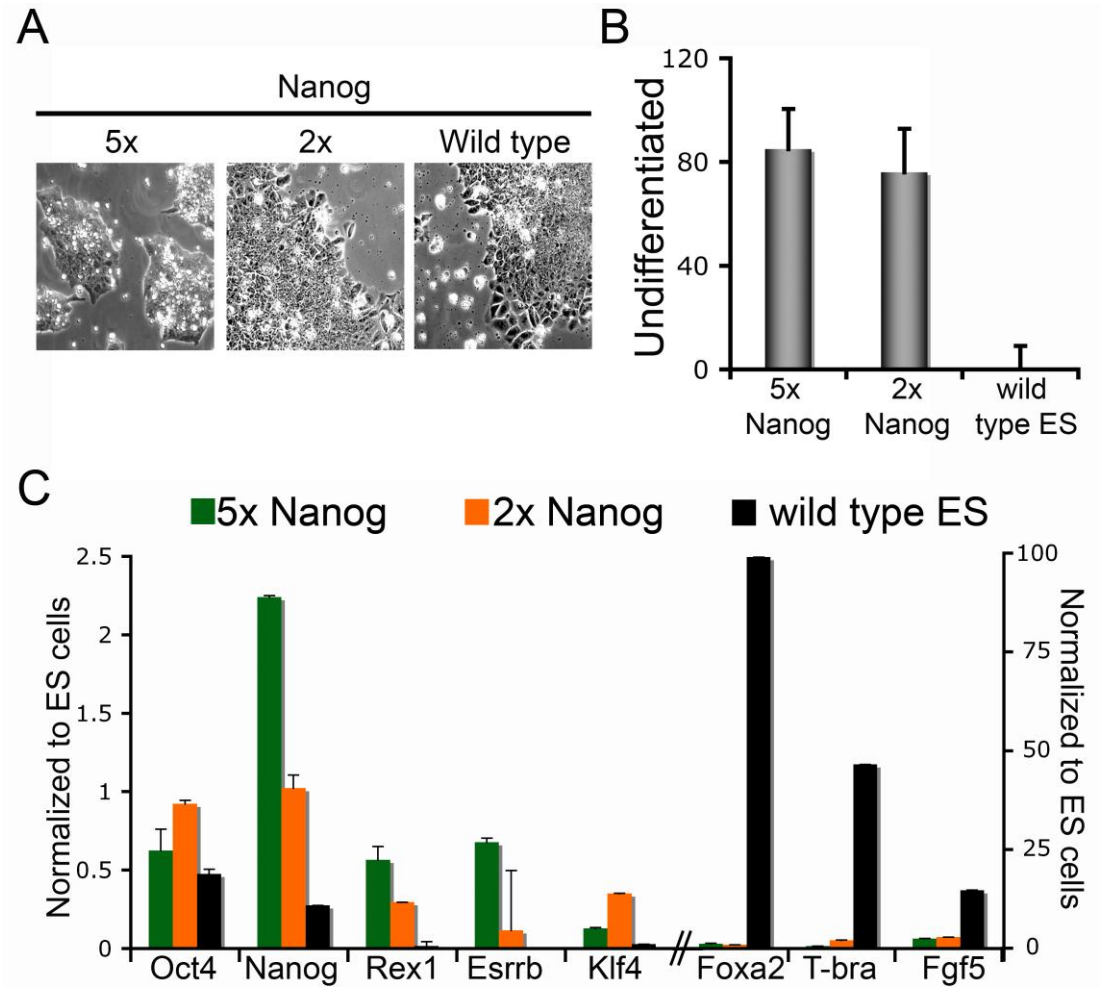


Figure 5. 6 Sustained Nanog expression blocks EpiSC differentiation.

(A) Brightfield images of cells overexpressing Nanog 5-fold (5x Nanog; EF4 cells), 2-fold (2x Nanog; RCN), and a control line with wild type Nanog levels (wild type ES; Oct4GiP), after culture in N2B27/Activin/bFGF for 8 passages.

(B) Cell lines that overexpress Nanog retain the ability to self-renew in stringent ES cell culture conditions; after 8 passages in N2B27/Activin/bFGF cells were replated in GMEMβ/FCS/LIF and colonies scored for expression of the ES cell marker, alkaline phosphatase (AP).

(C) Nanog overexpression prevents downregulation of ES cells specific transcription factors. qPCR showing mRNA levels of ES cell and EpiSC markers. qPCR analyses were determined relative to TBP and normalized to the ES cell level of 1.0.

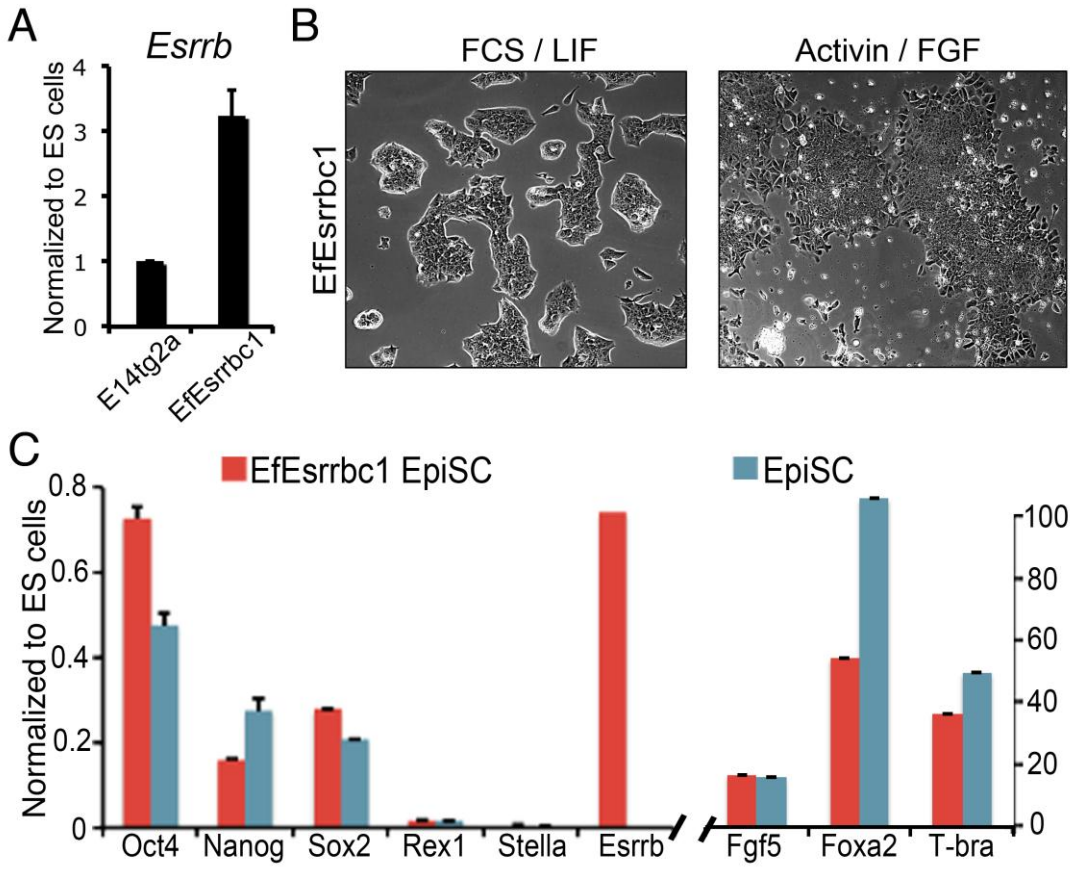


Figure 5. 7 Esrrb overexpression does not prevent transition to EpiSC pluripotency.

(A) qPCR for Esrrb in E14Tg2a and EfEsrrbc1 ES cells. The error bars are the SD of 3 technical replicates of one single experiment. EfEsrrbc1 ES cells were generated by Dr. Adam Yates.

(B) Brightfield images of EfEsrrbc1 cells cultured in GMEMβ/FCS/LIF or N2B27/Activin/FGF for 3 passages. Cells grown in GMEMβ/FCS/LIF display an ES cell morphology, whereas the cells grown in N2B27/Activin/bFGF display typical EpiSC morphology.

(C) Gene expression analysis of E14Tg2a and EfEsrrbc1 cells passaged in N2B27/Activin/bFGF for 5 passages. E14Tg2a and EfEsrrbc1 ES cells were plated at a density of 4 x 10^4 on fibronectin-coated plates and withN2B27/Activin/bFGF. Cells were passaged every 4-5 days using accutase and replated at a 1:10 ratio. qPCR analyses were determined relative to TATA box-binding protein (TBP) and normalized to the parental ES cells. All qPCR results are shown as the average of distinct experiments performed in at least 2 independent RNA preparations.

5.3 Reversion of EpiSC to an ES cell state by Esrrb expression

ES cells pluripotency can be restored in EpiSC by the overexpression of several transcription factors ES cell regulators such as Klf4, Klf2, Stat3, Nr5a receptors and Nanog (Guo and Smith, 2010; Guo et al., 2009; Hall et al., 2009; Silva et al., 2009; Yang et al., 2010). The overexpression of these factors alone is not sufficient to re-establish ES cell pluripotency, but must be accompanied by a switch into LIF containing media. A pre-requisite for the induction of an ES cell state is the removal of Activin and FGF (Hall et al., 2009). This suggests that in this context, extrinsic cues have a dominant effect over intrinsic factors on cell fate.

Esrrb is absent from EpiSC, but it is expressed in the undifferentiated ES cells (Figure 5.2; Figure 5.5). To test whether Esrrb can revert EpiSC to an ES cell phenotype, a system for episomal transduction and expression of cDNAs in EpiSC was used (Figure 5.8A). This system requires the cDNA of interest to be cloned into a plasmid containing the polyoma origin of replication. This allows extra-chromosomal replication in cells expressing the polyoma large T antigen (PyLT (Figure 5.8B)). An advantage of this expression system is that the plasmid does not integrate into the genome. Supertransfectable E14/T and TNG/T ES cells were differentiated into EpiSC, and subsequently, tested for their ability to be supertransfected (Figure 5.8). Since EpiSC propagate poorly at clonal density, a modified protocol for the supertransfection of the cells was established. The day following transfection the cells were dissociated, and seeded at a 7-fold higher density than used for ES cells. (Figure 5.8A; full protocol details can be found in

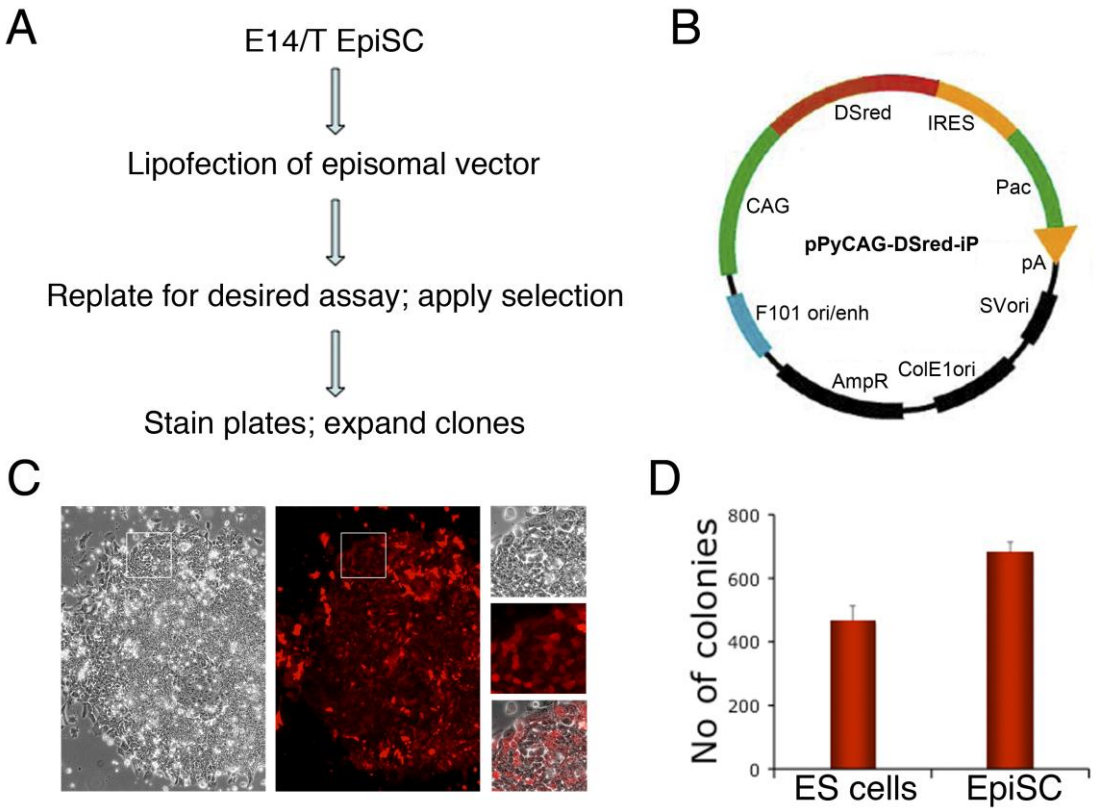


Figure 5. 8 Episomal expression system in EpiSC.

(A) Experimental strategy for the episomal vector expression in EpiSC.

(B) pPyCAGIP plasmid containing the polyoma origin of replication. cDNA is cloned directionally in place of the stuffer fragment within a transcription unit linked to the puromycin resistance gene (pac) through an IRES.

(C) Fluorescence and brightfield images of EpiSC with episomal expression of the vector pPyCAG-DSred-IP. The inset depicts a magnification of the boxed area in the left and middle images.

(D) Colony forming assays comparing episomal expression system in ES cells and EpiSC. 1 x 10^6 ES cells or EpiSC expressing PyLT were transfected with vector pPyCAG-DSred-IP in GMEMβ/FCS/LIF or N2B27/Activin/bFGF respectively. The following day the ES cells were collected with trypsin, while the EpiSC were harvested with accutase, and replated at a density of 4 x 10^4 in their respective media. The plates were stained for Leishman stain after 7 days and scored manually. The mean and SD of at least 3 independent experiments performed in triplicate are shown.

Chapter 2). Initial experiments assessed transfection of E14/T EpiSC using pPyCAG-DSRED-iP. Following 7 days of selection the vast majority of the cells expressed DSred, albeit expression was heterogeneous at the cellular level (Figure 5.6C). This is typical feature of the episomal expression system (Douglas Colby & Ian Chambers, personal communication). Importantly, the transfection efficiency observed for EpiSC was comparable to that obtained for ES cells (Figure 5.8D).

Nanog and Klf4 have been shown to reprogramme EpiSC to naïve pluripotency (Guo et al., 2009; Silva et al., 2009). Therefore, the abilities of Nanog, Esrrb and Klf4 to mediate the reversion of EpiSCs to an ES cell state were compared. Supertransfection of Nanog, Klf4 or Esrrb, coupled with a media switch from N2B27/Activin/Fgf to N2B27/2i/LIF, promoted EpiSC reprogramming to ground state pluripotency (Figure 5.9). It has recently been reported that Nanog can promote the reversion of EpiSC to an ES cell state in the complete absence of exogenous factors (Theunissen et al., 2011). Episomal expression of Nanog or Esrrb in EpiSC also allows reversion to ES cell pluripotency in the presence of FCS alone, an additive that others often eschew for undisclosed reasons (Figure 5.9). Strikingly, supertransfection of Nanog and Esrrb in EpiSC allowed reversion to ES pluripotency in the complete absence of exogenous growth factors (Figure 5.9). Interestingly, episomal expression of Nanog or Esrrb in EpiSC allows reversion to ES cell pluripotency in all conditions tested, whereas Klf4 could only reprogramme EpiSC in N2B272i/LIF (Figure 5.9; 5.10). Importantly, episomal expression of control plasmids, such as an empty vector, did not yield any revertant colonies (Figures 5.9; 5.10).

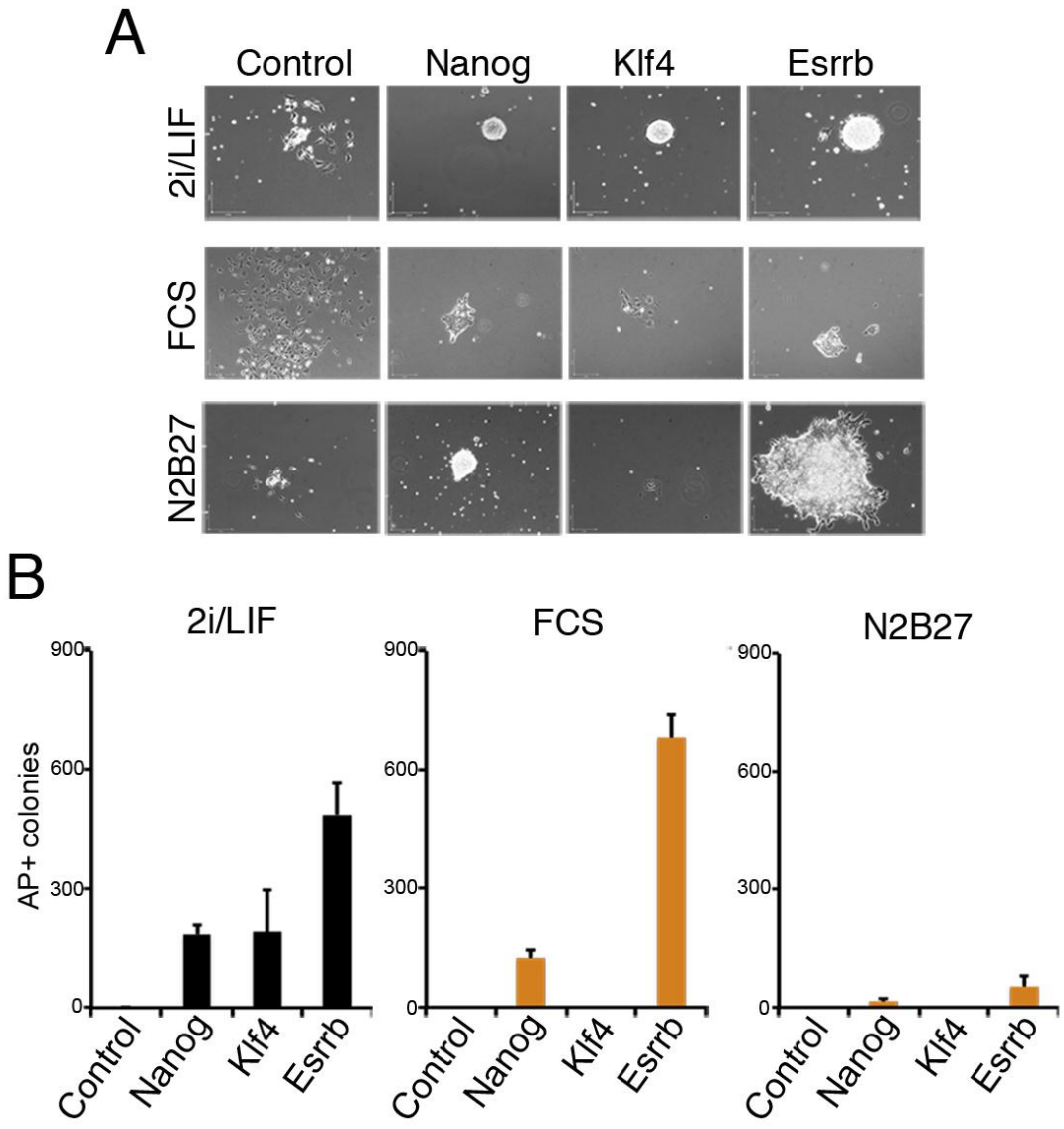


Figure 5. 9 Epi-iPS induction efficiencies by the episomal expression of Nanog, Klf4 or Esrrb in different media.

(A) Morphology of primary Epi-iPS colonies formed after transfection of the respective episomal vector and cultured in the indicated medium for 7 days. EpiSC expressing the polyoma large T-antigen protein were lipofected with episomal vectors containing empty vector, Nanog, Klf4 or Esrrb. The next day the cells were replated in the indicated media in the presence of puromycin selection.

(B) AP-positive colony formation by Epi-iPS cells. Experiment described in A was AP stained and the plates scored. The mean and SD of at least three independent experiment performed in triplicate are shown.

As previously mentioned, Nanog expression in EpiSC is lower than in ES cells (Figure 5.2B; 5.4). Consistent with this, *Nanog*:GFP is expressed at low levels in EpiSC, but can be detected by flow cytometry (Figure 5.10C). Therefore, increased *Nanog*:GFP expression could be used as an indicator of successful reprogramming to an ES cell state. Episomal expression of Nanog and Esrrb, coupled with a media switch to GMEMβ/FCS/LIF, resulted in the appearance of *Nanog*:GFP$^+$ colonies (Figure 5.10B). *Nanog*:GFP$^+$ colonies were not observed in cells that were transfected with Klf4 or control DSred plasmid (Figure 5.10B). Pecam1, a cell surface marker expressed in the inner cell mass (ICM)/ES cells and downregulated in the epiblast/EpiSC (Hayashi et al., 2008; Robson et al., 2001), was upregulated in with episomal expression of Nanog and Esrrb (Figure 5.10C).

Esrrb-induced Epi-iPS colonies were picked and expanded in conventional ES cell media. Expansion of the Epi-iPS clones was conducted in the absence of selection, since it has been shown in ES that absence of selection leads to episome loss (Douglas Colby & Ian Chambers, personal communication). Epi-iPS that had lost the plasmid were tested for their re-acquired sensitivity to puromycin and used for further studies. The expression of Nanog, Sox2, Klf4 and Tbx3 in Epi-iPS was restored to levels observed in ES cells, while Fgf5, an early marker of differentiation was downregulated (Figure 5.11A). Crucially, injection of Epi-iPS into blastocyst produced viable adult chimaeras, indicating that ectopic expression of Esrrb can restore the ability of EpiSC to colonise pre-implantation embryos (Figure 5.11B). Two Epi-iPS clones tested yielded high contribution adult chimaeras (Figure 5.11C).

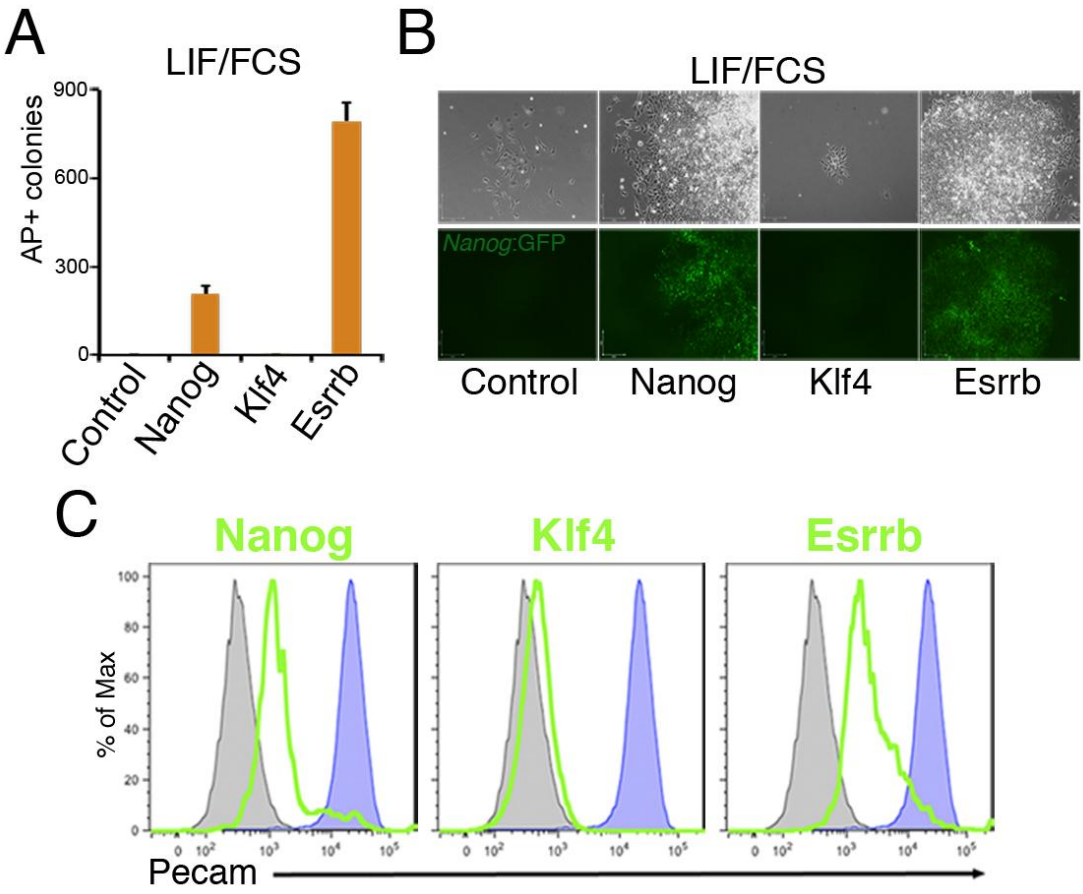
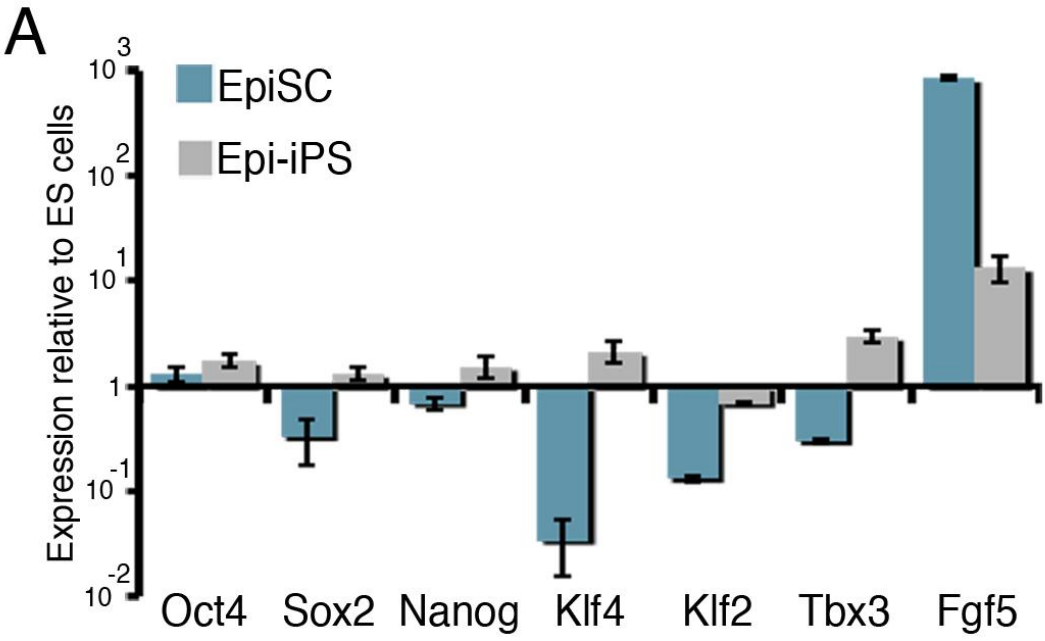


Figure 5. 10 Epi-iPS induction by the episomal expression of Nanog, Klf4 or Esrrb.

(A) Scoring of the AP stained plates following Epi-iPS induction by episomal expression of Nanog, Klf4 and Esrrb in GMEMβ/FCS/LIF. The mean and SD of at least three independent experiment performed in triplicate are shown.

(B) Morphology and *Nanog*:GFP expression of primary Epi-iPS colonies formed after transfection of the respective episomal vector and cultured in GMEMβ/FCS/LIF for 7 days.

(C) FACS analysis of Pecam1 expression 7 days after transfection of EpiSC with the indicated DNAs. TNG/T EpiSC (gray) and ES cells (blue) were used as controls.



B

Cell line	No. of born mice	Chimaeras
Epi-iPS C3	15	5
Epi-iPS C8	7	2

C

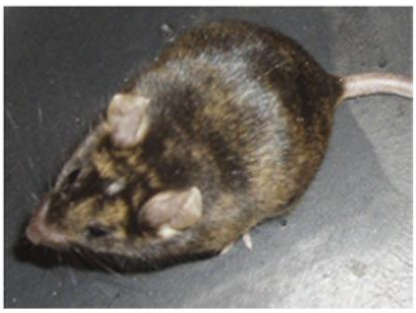


Figure 5. 11 Episomal Esrrb expression restores chimaera competency to EpiSC.

(A) mRNA expression in E14/T EpiSC and Epi-iPS colonies expanded in the absence of selection after episomal expression of Esrrb and medium switch into GMEMβ/FCS/LIF. Error bars: standard deviation of gene expression in 3 independent experiments.

(B) Esrrb induced Epi-iPS cell chimaera forming capacity. The number of adult chimaeras obtained from two independent blastocyst injections.

(C) Chimaeric mouse obtained from blastocyst injection of Esrrb-induced Epi-iPS.

It was then investigated whether stable integration of Cre-revertable plasmids could be used to reprogram EpiSC. In order to achieve efficient deletion, an EpiSC line was derived from the previously described ES cell line ROSA:Cre-ER^{T2} (RC), that constitutively express a tamoxifen inducible Cre recombinase (Cre-ER^{T2}) from the *ROSA26* (Chambers et al., 2007). The previously described loxP flanked Nanog expression plasmid (Chambers et al., 2007) was used to prepare derivative Esrrb/Klf4 plasmids (Figure 5.12A). Upon Cre-excision, the transcription factor ORF is deleted and GFP is expressed constitutively. Nucleofection introduced the Cre-revertable plasmids into the RC EpiSC, followed by selection in EpiSC media. The overexpression of the different factors in EpiSC was verified using real time PCR (Figure 5.12B). Cells were then challenged with a media switch to conventional ES media (LIF/FCS). The stable integration of loxP-flanked *Nanog*, *Klf4* and *Esrrb* transgenes into RC EpiSC yielded Epi-iPS colonies (Figure 5.12C). Under these conditions Esrrb displayed a higher efficiency than Nanog (5 fold) and Klf4 (9 fold) to reprogramme EpiSC (Figure 5.12C). Reverted clones were picked and expanded in conventional ES cell media in the presence of selection. Tamoxifen treatment of the reverted clones resulted in constitutive GFP expression, indicating successful deletion of *Esrrb* (Figure 5.13A). Flow cytometry analysis revealed that absence of GFP expression prior to tamoxifen treatment and the efficiency of deletion following a 24hr treatment with tamoxifen (Figure 5.13B, top panel). Pecam and c-kit, cell surface markers expressed in undifferentiated ES cells but that are downregulated in EpiSC were induced in Epi-iPS and remain unaltered following Cre-excision of the Esrrb transgene (Figure 5.13B, middle and bottom panels).

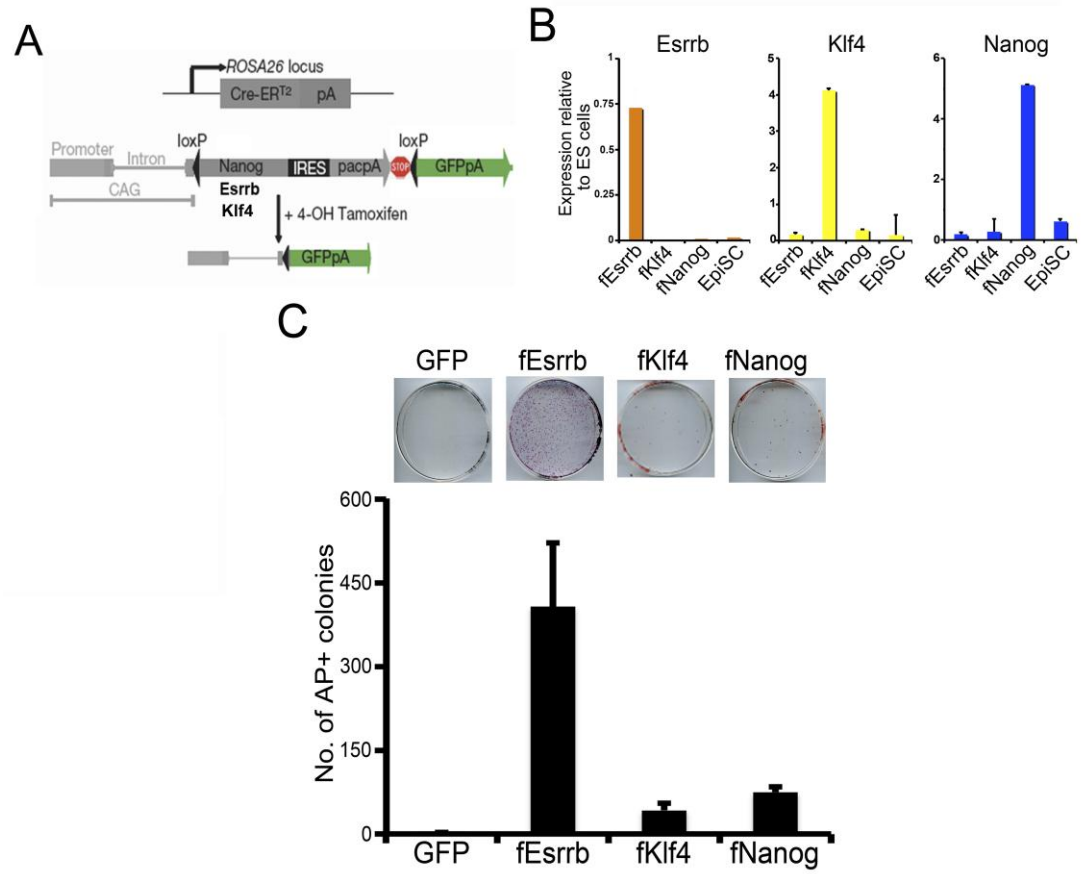


Figure 5. 12 Stable integration of Nanog, Klf4 or Esrrb allows the reversion of EpiSC into an ES-like state.

(A) Schematic diagram of the RC EpiSC line and the Cre revertible vectors that were used for stable integration of eGFP, Nanog, Klf4 and Esrrb.

(B) mRNA expression of Nanog, Klf4 and Esrrb in RC EpiSC, fEsrrb (RC + floxed Esrrb), fKlf4 (RC + floxed-Klf4) and fNanog (RC + floxed-Nanog) cultured in N2B27/Activin/bFGF. Error: Standard deviation of the mean in at least 2 independent experiments.

(C) Number of alkaline phosphatase positive colonies observed 7 days after replating 2 x 10^5 EpiSC stably transfected with Cre revertible GFP, Nanog, Klf4 and Esrrb vectors in N2B27/2i/LIF. Error: Standard deviation of the mean in 3 independent experiments. Representative AP stained plates are shown on top of the graph.

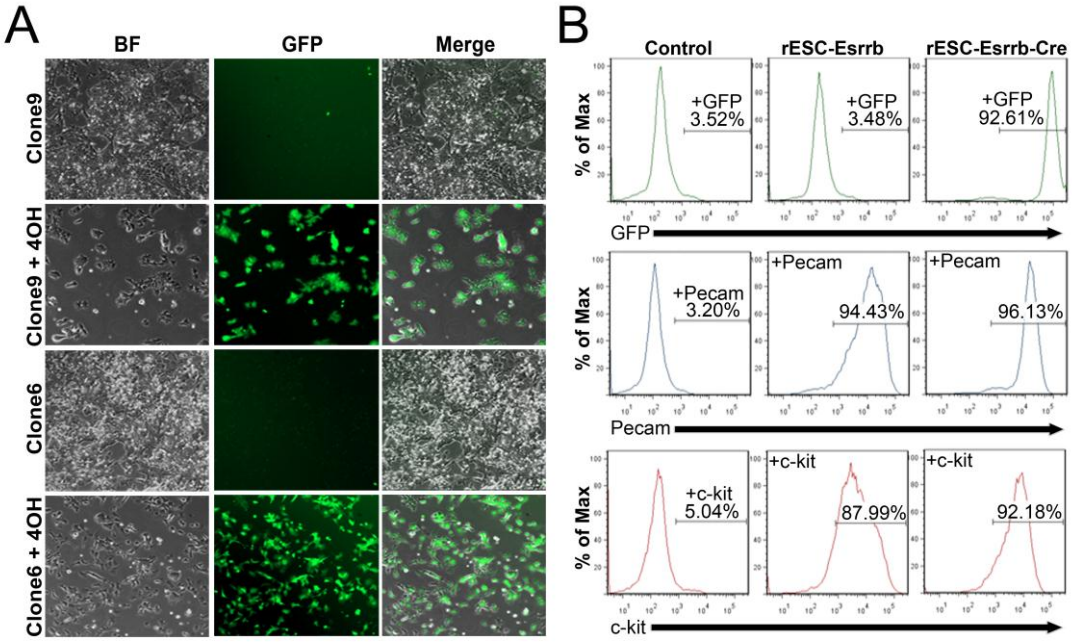


Figure 5. 13 Cre-excision of floxed-Esrrb.

(A) Two clones of the Esrrb reverted ES-like cells were treated with 1μM of Tamoxifen (Sigma) for 24h to Cre-excise the floxed Esrrb vector. Successful Cre-excision is demonstrated by the ubiquitous expression of eGFP. The first and third rows of panels show the untreated Epi-iPS clones (controls), meanwhile the second and fourth panels depict cells treated with 1μM Tamoxifen.

(B) Flow cytometry analysis on untreated and treated Epi-iPS for 24h with 1μM of Tamoxifen (top panels). The Epi-iPS were stained before and after Cre excision of Esrrb, for the cell surface markers Pecam (middle panel) and ckit (lower panel), which are markers absent in EpiSC but upregulated in ES cells.

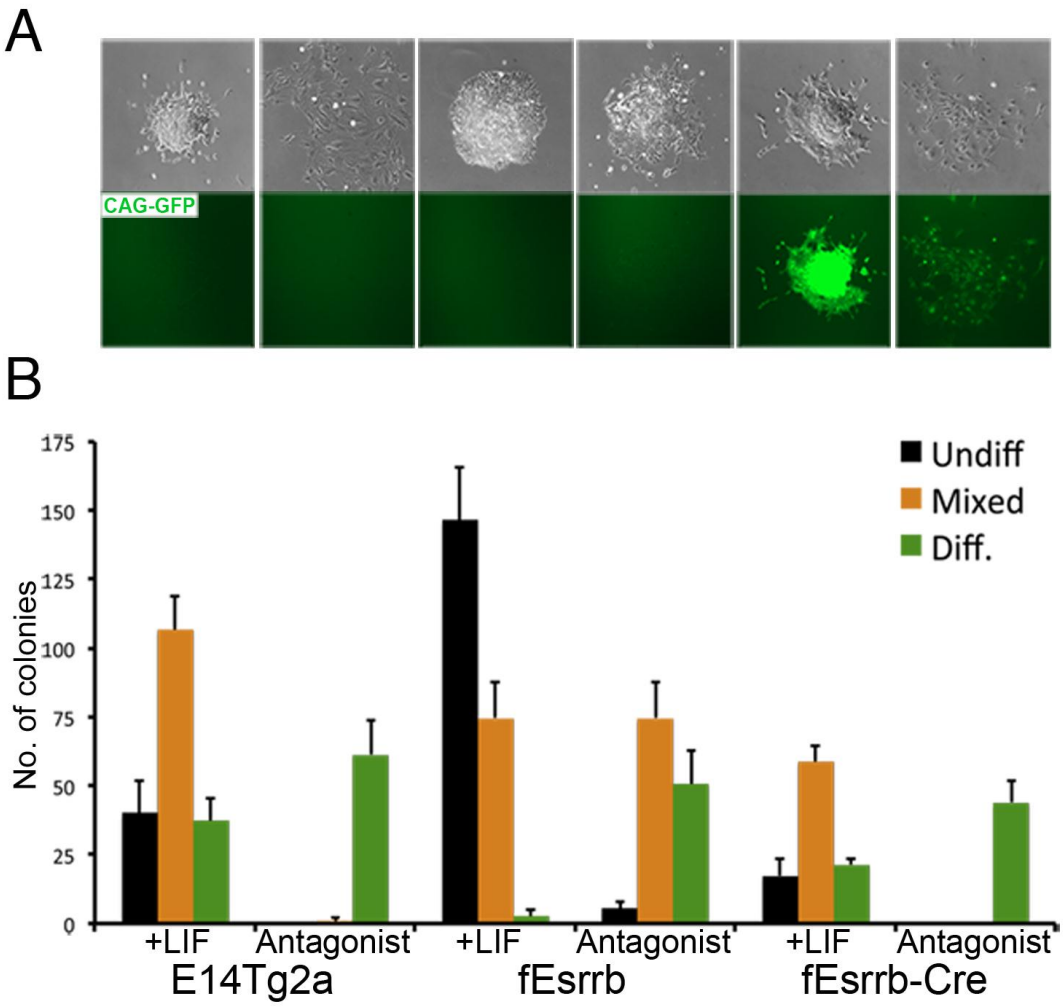


Figure 5. 14 Esrrb transgene is dispensable for Epi-iPS self-renewal.

(A) Morphology in E14Tg2a ES cells, fEsrrb Epi-iPS and f-Esrrb-Cre Epi-iPS plated at clonal density in GMEMβ/FCS in the presence of LIF or LIF antagonist.

(B) Quantification of the experiment described in A. Error bars are the standard deviation of the mean in 3 independent experiments.

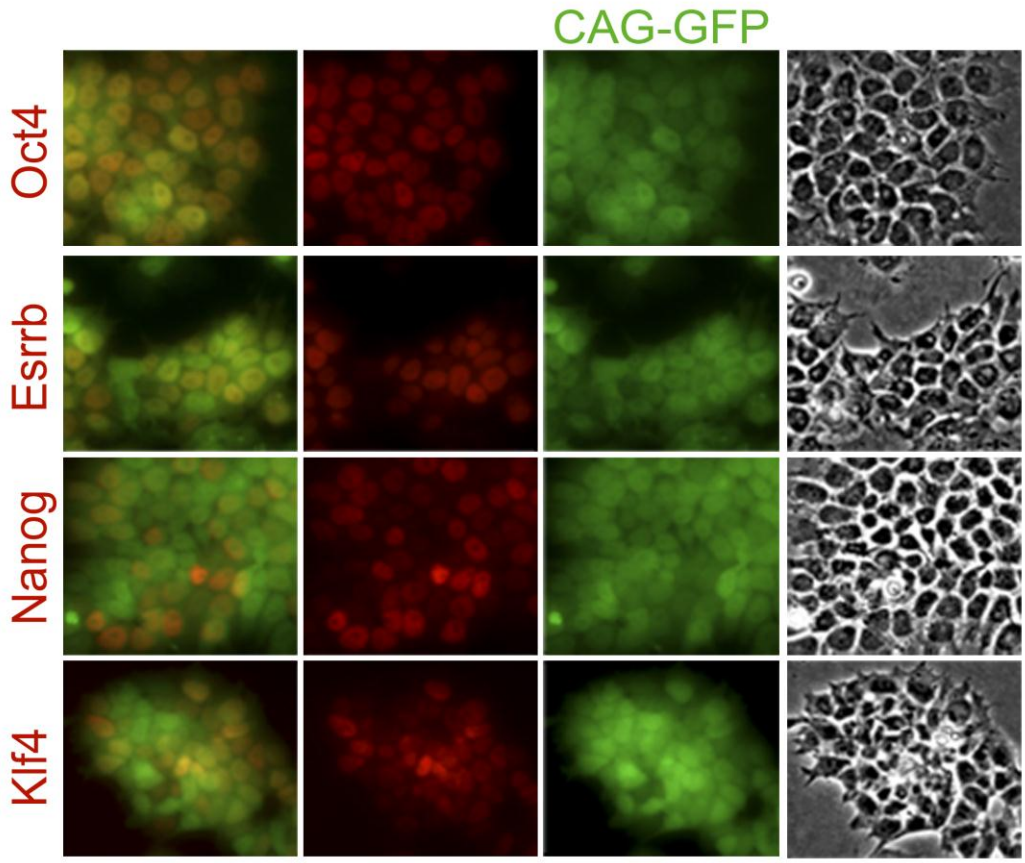


Figure 5. 15 Epi-iPS cre-revertants display heterogeneous expression of key pluripotency markers.

Immunohistochemistry for Klf4, Esrrb, Nanog, Sox2 and Oct4 proteins in Epi-iPS Cre-revertants. The Epi-iPS-Cre cells were plated at low density (5000 cells) into 4-well plates, the next day the cells were fixed using 4% PFA. The images were taken using an Olympus IX51 microscope and process using Volocity (Improvision) software.

The self-renewal of Esrrb-derived Epi-iPS showed reduced cytokine dependency before tamoxifen treatment, whereas cytokine dependence was restored in Cre-revertants (Figure 5.14). Similar to ES cells, Cre-revertants displayed heterogeneous expression of ES cell transcription factors Nanog, Klf4 and Esrrb (Figure 5.15).

5.4 Nanog-null EpiSC can be reverted back to naïve pluripotency

Next it was tested whether *Nanog*$^{-/-}$ EpiSC could be reprogrammed with defined factors (Figure 5.16A). Uncut, circular, plasmid DNA containing Nanog, Klf4 or Esrrb were lipofected into *Nanog*$^{-/-}$ EpiSC and following two days cells were transferred to N2B27/2i/LIF. Transient expression of Nanog and Esrrb, but not Klf4, combined with a media change to N2B27/2i/LIF, yielded Epi-iPS colonies (Figure 5.16B).

Further confirmation that Esrrb could reprogram EpiSC to an ES cell state in the absence of Nanog was obtained using a doxycycline-inducible expression system (Figure 5.17; 5.18; 5.19). ES cells in which expression of Nanog or Esrrb could be induced from the same genetic locus in a *Nanog*$^{-/-}$ background had been developed in the Chambers' lab (Figure 5.17; cells provided by Nicola Festuccia). A three-fold overexpression of Nanog or Esrrb compared to wildtype ES cells was obtained (Figure 5.17B, C). The 44iN and 44iE ES cells were converted into EpiSC by *in vitro* differentiation. 44iN and 44iE EpiSC were plated into GMEMβ/FCS/LIF in

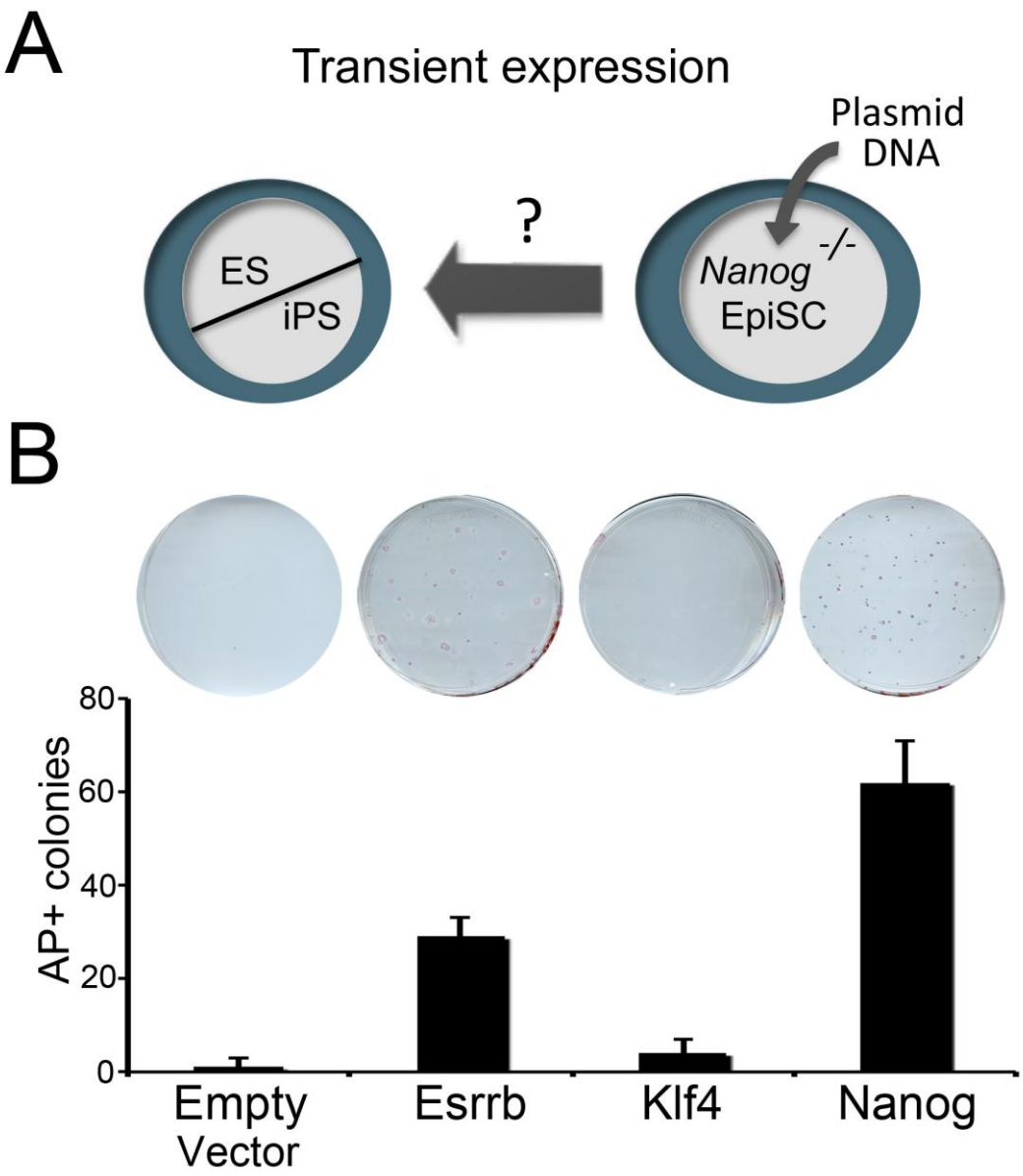


Figure 5. 16 Transient expression of Nanog or Esrrb mediates EpiSC reprogramming.

(A) Experimental strategy for EpiSC reprogramming using transient expression of defined factors.

(B) Transient expression of Nanog or Esrrb in EpiSC and a media switch to N2B27/2i/LIF promotes reversion to an ES cell state. Top panel: representative AP stained plates; Bottom panel: scoring of the AP stained plates.

the presence or absence of doxycycline and stained the plates for alkaline phosphatase after 7 days. Reverted colonies were obtained in the plates in which Nanog was induced. Interestingly, Epi-iPS colonies were also readily generated in the plates in which Esrrb was induced (Figure 5.18A). Using an inducible system allowed determination of the minimum exposure period necessary for the reprogramming of *Nanog*$^{-/-}$ EpiSC. Therefore, we plated the 44iN and 44iE EpiSC into LIF/FCS with different exposures periods of doxycycline. AP-positive colonies were obtained after as little as 24 hour induction of Nanog or Esrrb, with a clear positive correlation between the period of exposure and the number of reverted colonies (Figure 5.18A, B). Importantly, no reverted colonies were obtained in plates that were not induced with doxycycline (Figure 5.18A, B). Induced 44iN displayed a higher number of reverted colonies than the 44iE, suggesting that Nanog was more efficient than Esrrb in reprogramming of Nanog-null EpiSC (Figure 5.18A, B). Esrrb-induced reverted *Nanog*$^{-/-}$ clones were picked and expanded in conventional ES cell media in the absence of doxycycline. Importantly, the majority of the 44iE Epi-iPS cells had reacquired an ES cell morphology and re-expressed *Nanog*:GFP to an ES cell level (Figure 5.19A). Moreover, the 44iE Epi-iPS had reacquired the ability to self-renew in stringent ES culture conditions, such as BMP/LIF and 2i/LIF (Figure 5.19B). Gene expression analysis revealed that the 44iE Epi-iPS had downregulated the early marker of differentiation Fgf5, and re-acquired expression of Sox2, Klf4 and Tbx3 to ES cell levels (Figure 5.19C). Finally, when these cells were injected back into blastocyst-stage embryos they produced viable adult chimaeras (Figure 5.19D, E).

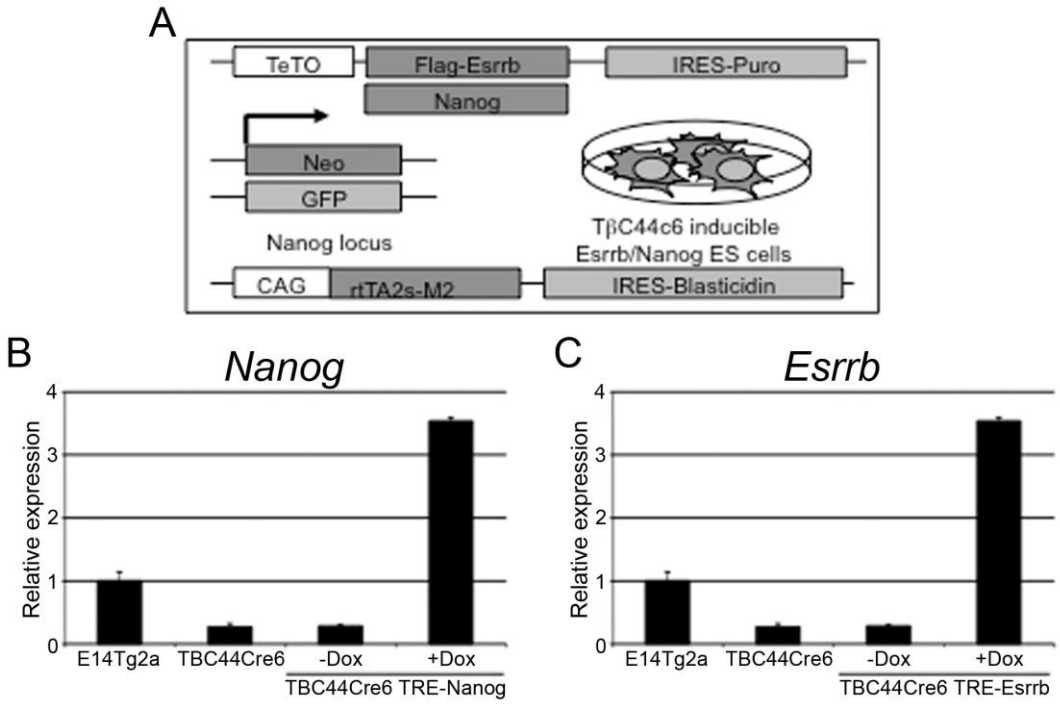


Figure 5. 17 Nanog-null ES cells were engineered to express Nanog or Esrrb in a doxycycline inducible manner.

(A) Scheme depicting the genetic modifications in the doxycycline-inducible 44iN and 44iE ES cells. ES cell lines (44iN and 44iE) were generated by Nicola Festuccia).

(B) qPCR showing the total mRNA levels for Nanog in 44iN, parental line TβC44Cre6 and E14Tg2a ES cells.

(C) qPCR showing the total mRNA levels for Esrrb in 44iE, parental line TβC44Cre6 and E14Tg2a ES cells.

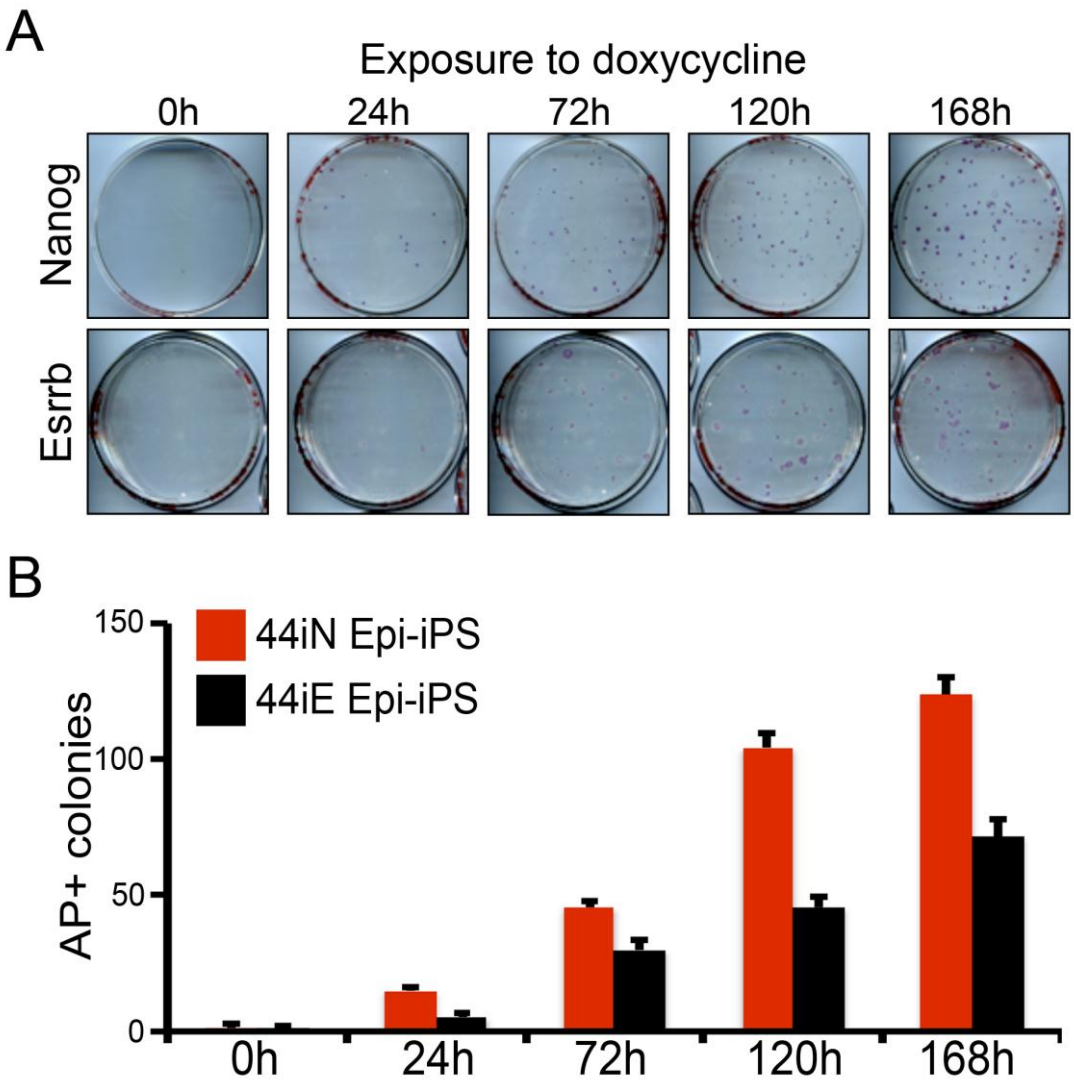
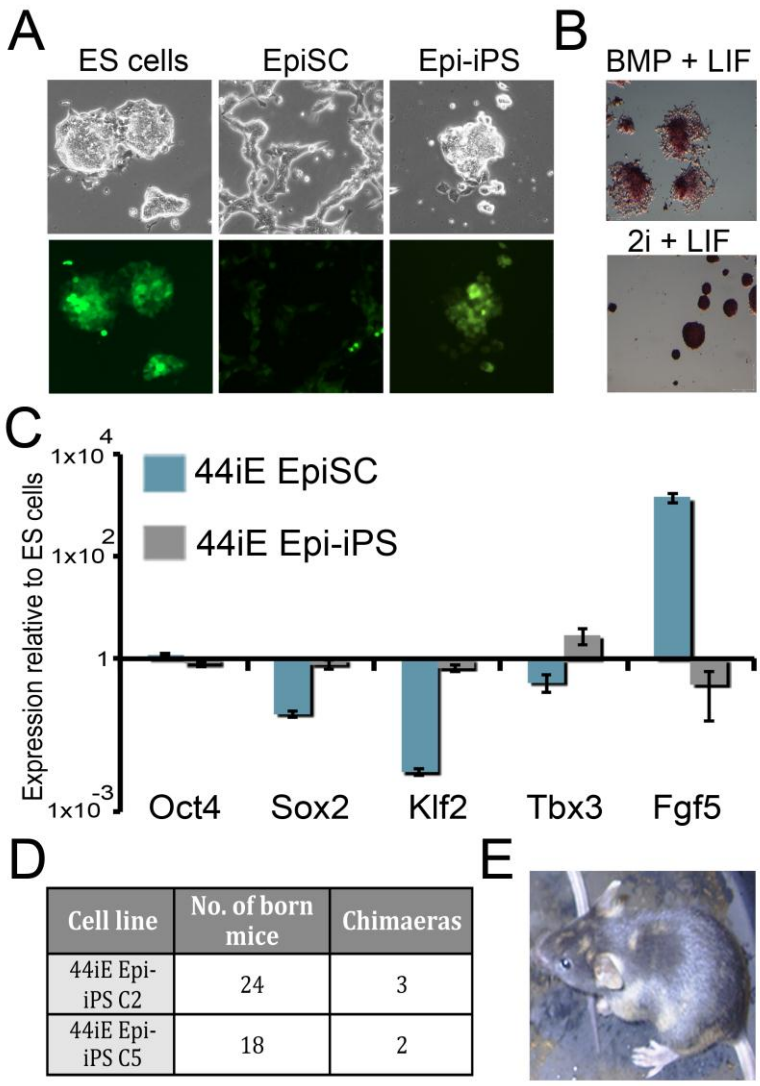


Figure 5. 18 *Nanog*$^{-/-}$ EpiSC can be reverted back to an ES cell state using doxycycline-inducible Nanog or Esrrb.

(A) Nanog-null EpiSC that are inducible for Nanog or Esrrb can be reverted back to an ES-like state. 44iE and 44iN EpiSC were replated in 9cm dishes at a density of 5 x 10^4 in GMEMβ/FCS/LIF in presence of doxycycline for an array of different exposure periods (0h, 24h, 72h, 120h and 168h). After 7 days of culture the plates were stained for alkaline phosphatase.

(B) Graph depicting the reprogramming efficiency of 44iE and 44iN EpiSC with different exposures periods of doxycycline described in A. The plates were stained for alkaline phosphatase after 7 days and scored manually. The mean and SD of at least 3 independent experiments performed in triplicate are shown.



Cell line	No. of born mice	Chimaeras
44iE Epi-iPS C2	24	3
44iE Epi-iPS C5	18	2

Figure 5. 19 Doxycycline-inducible Esrrb restores ES cell pluripotency to Nanog-null EpiSC.

(A) Bright field and *Nanog*:GFP images of 44iE ES cells, EpiSC and Epi-iPS cells.

(B) Reverted Nanog-null ES-like cells can self-renew in serum free conditions in N2B27 supplemented either with BMP/LIF or 2i/LIF.

(C) mRNA expression in 44iE EpiSC and Epi-iPS colonies expanded in the absence of doxycycline. Error bars: standard deviation of gene expression in 3 independent experiments.

(D) 44iE Epi-iPS cell chimaera forming capacity. The number of adult chimaeras obtained from two independent blastocyst injections.

(E) Chimaeric mouse obtained from a blastocyst injection with the Esrrb-induced Epi-iPS (*Nanog*$^{-/-}$).

5.5 Discussion

ES cells and EpiSCs express the core pluripotency factors Oct4, Sox2 and Nanog and this is consistent with their tissue of origin. Nevertheless, ES cells and EpiSCs are two distinct pluripotent populations since they show differences in their growth factor responsiveness, morphology and their abilities to undergo clonal propagation and re-colonize embryos. It is possible to harness this differential growth factor responsiveness to convert ES cells into EpiSCs simply by switching the media composition and passaging. Alternatively, EpiSC can be reverted back to ES cell pluripotency through the individual overexpression of a small number of transcription factors. Although the inter-conversion of ES cells and EpiSCs has been documented, further analysis of the changes that occur during such transitions may be informative. The current chapter deals with the transcription factor requirements for the transitions between ES cells and EpiSCs.

5.5.1 Conversion of ES cells into EpiSCs

Several studies have placed Nanog at the cusp of the pluripotency network regulating ES cell self-renewal (Chen et al., 2008; Ivanova et al., 2006; Loh et al., 2006). Moreover, Nanog levels show a solid positive correlation with the self-renewal efficiency of ES cells (Chambers et al., 2003; Chambers et al., 2007). It has been shown that following the specification of the naïve epiblast, *Nanog* is downregulated prior to implantation (E4.5) and subsequently re-expressed at the egg cylinder stage (Chambers et al., 2003; Hart et al., 2004; Osorno et al., 2012). Analyses of peri-implantation embryos using *Nanog*:GFP mice indicate that, indeed, Nanog is first downregulated and then upregulated in peri-implantation embryos

(see Section 5.2). This transition was recapitulated *in vitro* by culturing ES cells in media supplemented with growth factors that support EpiSC self-renewal. An early event during this transition is the reduction in Nanog levels. Interestingly, several prominent Nanog target genes, such as *Esrrb* and *Klf4* (Festuccia et al., 2012), are downregulated during the early stages of differentiation (see Section 5.2). It is possible that the changes in expression of *Esrrb* and *Klf4* are a direct consequence of *Nanog* dowregulation. Indeed, when Nanog overexpressing cells (Chambers et al., 2003; Chambers et al., 2007) are cultured in EpiSC media a negative correlation was found between the Nanog levels and the ability of ES cells to commit to an EpiSC state. Nanog overexpressors resisted the upregulation of differentiation genes and failed to downregulate ES cell specific markers such as *Rex1*, *Esrrb* and *Klf4*. Even after prolonged passage in EpiSC media, Nanog overexpressors retained the ability to self-renew in ES cell media. Although Nanog overexpressors cultured after prolonged passage in N2B27/Activin/FGF retain the ability to self-renew in GMEMβ/FCS/LIF, the colony forming capacity is lower than that obtained with its ES cells counterparts (see section 5.2). This observation may be due to a combination of factors: (1) low plating efficiency, (2) higher cell death and/or (3) reduced self-renewal efficiency. Future work will no doubt help clarify the functional differences between the cell types.

It has been shown that Klf4 overexpression does not block differentiation of ES cells into EpiSCs (Guo et al., 2009). Moreover, Esrrb overexpressing cells could not recapitulate the effect of sustained Nanog expression during the induction of EpiSC (see Section 5.2). This feature seems unique to Nanog since cells overexpressing Esrrb or Klf4 do not resist conversion into EpiSCs. Therefore, Nanog

levels seem to play a key role in the specification of the pluripotent state of the cells. The mechanisms through which Nanog defines the pluripotent state are likely to involve Nanog target genes. It is likely that the *in vivo* and *in vitro* downregulation of Nanog results in the removal of Nanog target genes that are essential for maintaining naïve pluripotency.

5.5.2 Esrrb reverts EpiSC to ES pluripotency

Sox2 and Nanog are expressed at lower levels in EpiSC when compared to ES cells (Han et al., 2010; Osorno and Chambers, 2011). Several transcription factors reported to be involved in ES cell self-renewal are not expressed in EpiSC, these include Esrrb, Klf4, Klf2 and Nr5a2 (Guo and Smith, 2010; Ivanova et al., 2006; Jiang et al., 2008; Niwa et al., 1998). Furthermore, EpiSC express lower levels of Nanog, Tbx3, LIF receptor and Stat3 (Figure 5.2; Figure 5.4; (Han et al., 2010; Yang et al., 2010). Interestingly, the overexpression of Klf4, Klf2, Nr5a2 and Stat3, combined with a media switch, can revert EpiSC to ES cell pluripotency (Guo and Smith, 2010; Guo et al., 2009; Hall et al., 2009; Yang et al., 2010). Naïve pluripotency can also be re-instated by elevating the levels of Nanog in EpiSCs. These observations prompted us test the ability of Esrrb to reprogramme EpiSCs to naïve pluripotency. Strikingly, side-by-side comparison revealed that Esrrb surpasses Nanog and Klf4 in the efficiency to induce ES cell pluripotency in EpiSC. Interestingly, Klf4 overexpression was only able to reprogramme EpiSC to ES cell pluripotency in the presence of exogenous LIF and the combined inhibition of GSK3 (CHIR99021/MEK (PD0325901) (Han et al., 2011; Silva et al., 2009). In contrast, Nanog and Esrrb generated Epi-iPS in all culture conditions tested. Moreover, Esrrb and Nanog

(Theunissen et al., 2011) can relieve the requirement for exogenous LIF during the reversion of EpiSCs to naïve pluripotency. Strikingly, episomal expression of Nanog or Esrrb induced the formation of Epi-iPS in the complete absence of exogenous growth factors. This indicates that Nanog and Esrrb are potent inducers of ES cell pluripotency in EpiSC. On the other hand, the limited ability of Klf4 to reprogramme EpiSC suggests that this transcription factor cannot re-instate ground state pluripotency alone, but rather may need the presence/absence of downstream targets of GSK3, MEK or both. The above results suggest that Esrrb and Nanog are functionally similar in the context of EpiSC reprogramming. Such a functional overlap could be caused by the activation of a similar transcriptional programme during the induction of naïve pluripotency.

5.5.3 Esrrb can reverts *Nanog*$^{-/-}$ EpiSC to ES pluripotency

It was demonstrated in Chapter 4 that Nanog is not essential for the derivation and maintenance of EpiSC. To consolidate the notion that Esrrb can functionally substitute for Nanog, we tested the ability of Esrrb to reprogramme *Nanog*$^{-/-}$ EpiSC. Strikingly, Esrrb was able to restore naïve pluripotency in cells genetically depleted of Nanog. This is in stark contrast to the observation that Klf4, another Nanog target gene (Festuccia et al., 2012), is unable to reprogramme Nanog$^{-/-}$ cells. This reinforces the notion that Esrrb shows a high degree of functional overlap with Nanog. Interestingly, in the context of reprogramming in a Nanog-null background, Esrrb showed a lower reprogramming efficiency of EpiSC than Nanog. Together these observations suggests that Esrrb and Nanog act cooperatively during reprogramming to induce naïve pluripotency. It would be

interesting to see whether Nanog can reprogram cells in the complete absence of Esrrb.

Chapter 6

General Discussion

The ability of a single cell to generate every cell type in the adult organism has been termed as pluripotency. *In vivo*, pluripotent cells exist transiently in the epiblast of the developing embryo and in rare tumour cells. Pluripotent cells have been isolated from the pre-implantation and post-implantation epiblast (Brons et al., 2007; Evans and Kaufman, 1981; Martin, 1981; Tesar et al., 2007). The *in vitro* derivatives of these, ES cells and EpiSCs respectively, also express the core pluripotency factors Oct4, Sox2 and Nanog.

6.1 Cellular heterogeneity

It has recently been shown that chemical inhibition of GSK3 (CHIR99021) and MEK (PD0325901), combined with stimulation with LIF (2i/LIF), stabilizes the pluripotency network and promotes robust self-renewal (Ying et al., 2008). It has been hypothesized that perturbations that compromise the stability of this network may prime ES cells towards differentiation (Ying et al., 2008). Relevant to this is the finding that Nanog fluctuates between states of high Nanog expression, associated with high self-renewal efficiency, and low Nanog expression, associated with increased differentiation propensity (Chambers et al., 2007). Together these observations postulate Nanog as the key factor mediating the stability of the pluripotency network. Consistent with this, it has been reported that FGF/ERK signaling is necessary to prime ES cells for differentiation (Burdon et al., 1999;

Kunath et al., 2007; Stavridis et al., 2007) and it has been shown that this signaling negatively regulates Nanog expression (Hamazaki et al., 2006). Furthermore, MEK inhibition (PD184352) promotes the upregulation of *Nanog*, thus promoting self-renewal (Silva et al., 2009; Ying et al., 2008). In this context, Chapter 3 provides new insight into the role of FGF/ERK signaling in the induction of heterogeneous Nanog expression. During the early stages of differentiation, FGF/ERK signaling is required to destabilize the pluripotency network, thereby generating a balanced $Nanog^{high}/Nanog^{low}$ population. To clarify whether ERK signaling promotes differentiation through Nanog modulation, an obvious experiment would be to test the effects of MEK inhibition in Nanog-null ES cells. If the proposed model holds true one would predict that, in the absence of Nanog, MEK inhibition would not enhance self-renewal.

6.1 Gene regulatory networks governing pluripotent cell states

Genetic studies have demonstrated that the expression of Oct4, Sox2 and Nanog, is essential for the establishment of pluripotency in the embryo. Oct4 and Sox2 have been shown to be necessary for the maintenance of pluripotency in ES cells (Masui et al., 2007; Niwa et al., 2000), whereas Nanog expression is dispensable (Chambers et al., 2007). However, the overexpression of Nanog in ES cells confers cytokine-independent self-renewal (Chambers et al., 2003). Oct4, Sox2 and Nanog are also expressed in the pluripotent postimplantation epiblast and its *in vitro* derivatives (EpiSCs). However, the role of Oct4, Sox2 and Nanog in primed pluripotency remains largely unknown. The work in Chapter 4 shows that, prior to

somitogenesis, expression of the core pluripotency factors, Oct4, Sox2, and Nanog, is regionalized. It is puzzling that in the E7.5 embryo, where pluripotency is a general property of the epiblast, these genes are not ubiquitously expressed. Somatic pluripotency is lost in the mouse embryo around E8.5 (Damjanov et al., 1971), however, the precise timing and mechanism involved in this process has not yet been defined. In Chapter 4, it is shown that pluripotency is extinguished at the onset of somitogenesis, coincident with reduced expression and chromatin accessibility of *Oct4* and *Nanog*. Interestingly, pluripotency tracks the *in vivo* level of Oct4, but this correlation does not hold true for Nanog. Indeed, ectopic Oct4 expression in somitogenesis-stage tissue provokes rapid reopening of *Oct4* and *Nanog* chromatin, Nanog re-expression and resuscitation of moribund pluripotency. This competence to re-activate the pluripotency network upon enforced Oct4 expression is gradually lost with the progression of embryonic development. Intriguing is the observation that responsiveness to ectopic Oct4 varies between distinct tissues. This could be due to the presence/absence of other key pluripotency regulators. It would be interesting to determine whether Oct4-mediated reactivation of the pluripotency network is dependent on Sox2 expression. Additionally, one can ask whether the enforced expression of Nanog can reactivate the pluripotency network in somitogenesis stage embryos.

6.3 Lessons from EpiSC

ES cells and EpiSCs differ in several aspects, such as morphology, clonal propagation abilities and their requirements for exogenous factors to promote self-renewal. Moreover, ES cells possess the ability to re-colonize pre-implantation

embryos. EpiSCs, unless selected for rare subpopulations resembling very early postimplantation epiblast, are unable to contribute to chimeras in blastocyst injection or morula aggregation (Han et al., 2010). EpiSCs are particularly interesting because they share many attributes with human ES cells. For instance, the culture conditions used to derive and maintain EpiSC were identical to the routine culture conditions of hES cells. The observation that the same signals promote self-renewal across species suggests that the pathways to pluripotency may be evolutionary conserved. Furthermore, transcriptional analysis also suggests that EpiSC are closer to hES cells than to mES cells (Tesar et al., 2007). Although hES cells are derived from blastocyst stage embryos, the previous observations strongly argues in favour of the notion that hES cells correspond to a more developmentally advance pluripotent state, similar to the post-implantation mouse epiblast. It remains to be seen whether authentic "human ES cells", reminiscent of the mouse counterpart, can be isolated.

The functional differences between ES cells and EpiSC can also be harvested to further our understanding of the pluripotent state. For instance, ES cells can be converted into EpiSC by a simply media switch (Guo et al., 2009). The work presented in Chapter 5 indicates that during this conversion several ES cells specific factors are downregulated. Furthermore, it has been shown that Nanog levels are critical for the specification of the pluripotent state of the cells. This is consistent with the proposed role of Nanog as modulator of network activity. On the other hand, EpiSC can be reverted back to ES cell pluripotency through the overexpression of a small number of transcription factors (Guo and Smith, 2010; Guo et al., 2009; Hall et al., 2009; Silva et al., 2009; Yang et al., 2010). Interestingly,

the majority of the factors reported to reprogramme EpiSC also have a known function in ES cell self-renewal. The work presented in Chapter 5 shows that the Nanog target-gene, Esrrb, is a potent inducer of ES cell pluripotency in EpiSC. Side-by-side reprogramming experiments revealed that Nanog and Esrrb surpass Klf4 in the ability to generated Epi-iPS. Such an experimental approach is potentially very useful for the identification and characterization of additional key factors regulating pluripotent cell identity.

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